DOCTOR OF PHILOSOPHY

Investigation of the role of p53 in protecting cancer cells against PLK1 inhibitors

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Award date:
2017

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A thesis submitted for the degree of

Doctor of Philosophy

University of Dundee

August 2017

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Abbreviations

aa	Amino acid
APC/C	Anaphase Promoting Complex/ Cyclosome
APS	Ammonium persulfate
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine triphosphate
ATR	Ataxia-Telangiectasia and Rad3 Related
BAX	Bcl-2 Associated X Protein
Bcl	B Cell Lymphoma
BH3	Bcl-2 Homology Domain 3
BrdU	Bromodeoxyuridine
Bub1	Budding uninhibited by benzimidazoles 1
BubR1	Budding uninhibited by benzimidazole-related 1
CBP	CREB-binding protein
CDC	Cell division cycle
CDE	Cell cycle-Dependent Element
CDK	Cyclin Dependent Kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CHR	Cell cycle genes Homology Region
C-terminus	Carboxyl terminus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase

DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
Ect2	Epithelial cell transforming 2
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide Exchange Factor
h	Hour
H_2O_2	Hydrogen Peroxide
HIPK2	Homeodomain Interacting Protein Kinase 2
HCl	Hydrochloric Acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinases
kDa	Kilo-Dalton
LB	Lysogeny broth
MAPK	Mitogen activated protein kinases
MDM2	Murine Double Minute 2
MPM-2	Mitotic Protein Monoclonal #2
Myt1	Myelin Transcription Factor 1
Nek	NIMA (Never In Mitosis Gene A) - Related Kinase
N-terminus	Amino-terminus

NudC	Nuclear Distribution C
Orc2	Origin recognition complex 2
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP Ribose Polymerase
PBD	Polo Box Domain
PBIP1	Polo-Box Interacting Protein 1
PBS	Phosphate-buffered saline
PICH	PLK1-Interacting Checkpoint Helicase
PLK	Polo-like Kinase
pRB	Retinoblastoma protein
PUMA	p53 Up-regulated Modulator of Apoptosis
RCF	Relative centrifugal force
RING	Really Interesting New Gene
RNA	Ribonucleic acid
SCF	Skp, Cullin. F-box Containing Complex
SDS	Sodium dodecyl sulfate
STLC	S-Trityl-L-cysteine
SV40	Simian Virus 40
TAE	Tris-acetate-EDTA
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

Acknowledgements

Firstly I would like to thank Dr David Meek for the opportunity to complete my PhD in his lab. He has provided an excellent environment in which to work, and I thank him for all the support, guidance and good humour he has offered me. He has been an excellent supervisor throughout my PhD (even when he is being grumpy) and I wish him all the best with his new adventure. I would also like to acknowledge Dr Frances Fuller-Pace for her scientific input, kindness and for keeping David in line! Next, I would like to thank Dr Adrian Saurin who very kindly offered his expertise in mitosis and microscopy. Without his input I would not have made many of the findings that are presented in this thesis. I would also like to thank Magda and Giulia from the Saurin lab for assistance with microscopy.

I would next like to thank all the lovely people I have met and friends I have made from the Division of Cancer Research. You have all made me feel at home in Dundee and I will miss you all. A big thank you also goes to the past and present members of the Meek and FFP labs who helped me find my feet when I first arrived. In particular, I want to thank my amazing lab mates, Anna, Bianca and Maryam. They have become very good friends throughout this journey, and I am very grateful that they have been supportive, helpful and generally just great people to work with. I would definitely not have enjoyed my time as much without them, and I will be very sad to not see them every day as we all move on to the next step. If in my next job I work with people even half as wonderful as them I will count myself very lucky. I look forward to some future holidays to see them wherever they end up next though!

Without funding this work would not have been possible, so I would like to thank the MRC for funding this project. I also wish to thank all those involved in the ITTP

scheme, as I have thoroughly enjoyed spending a week in Leicester every year to learn about toxicology and having the opportunity to socialise with some lovely people.

I would also like to thank my family for their support, and my sister Julie for providing me with a home whilst I have been in Dundee. Also, thanks to Cassie and Matthew who I have enjoyed weekly dinners with throughout my time here. It's been nice to be reunited with some old school friends. Finally, I would like to thank my boyfriend, Simon, who has been very supportive throughout the past four years even though I 'ran away from him' to live in Dundee. I appreciate that he has made weekend sacrifices with the amount of football he plays in order to come and visit me. Hopefully we will be living in the same place in the not too distant future.

Declaration

I hereby declare that I am the author of this thesis; that, unless otherwise stated, all references cited have been consulted by me; that the work of which this thesis is a record has been carried out by me, and it has not been previously accepted for a higher degree.

Signed

Linda Smith August 2017

I hereby declare that Linda Smith has completed the work presented in this thesis under my supervision. I confirm that she has fulfilled the conditions of the relevant Ordinance and Regulations of the University of Dundee, thereby qualifying her to submit this thesis in application for the degree of Doctor of Philosophy.

Signed

Dr David W. Meek August 2017

Abstract

The protein kinase Polo-like kinase-1 (PLK1) is an essential driver of mitosis and is oncogenic when expressed at elevated levels. Therefore, a number of inhibitors of PLK1 have been developed as potential anti-cancer therapeutic agents. While these inhibitors undergo clinical evaluation there has been evidence to show that they are less effective in killing cancer cells expressing the transcription factor p53. Normally, p53 is a potent tumour suppressor that has a classical role in orchestrating inhibition of cancer cell growth. However, under some circumstances p53 appears to protect cancer cells, due to cells undergoing a reversible growth arrest. This may allow cells to recover post-treatment. Here, it has been shown that a proportion of p53 competent cells treated with PLK1 inhibitors undergo a G1/S arrest. This is mediated by p53 and may offer protection to these cells. Application of PLK1 inhibitors also gives rise to DNA damage, induces p53, promotes increased p53 phosphorylation, and causes activation of downstream targets, such as p21. Inhibiting the DNA damage-activated protein kinases ATM and ATR prior to treatment with PLK1 inhibitors alleviates the activation of p53. Interestingly, the resulting duration of mitotic arrest from inhibition of PLK1 is markedly shorter in cells that are p53 competent. Study of the centrosomes after PLK1 inhibition highlighted that centrosome separation is more greatly impaired in cells deficient of p53. Furthermore, use of an inhibitor of Eg5, a motor protein involved in centrosome separation, resulted in similar phenotypes to that observed with inhibitors of PLK1. This suggests p53 has a potentially novel role in centrosome separation and has highly important implications for treatment with PLK1, and perhaps Eg5, inhibitors. This work suggests that screening of individual patients for the p53 status of their cancer could be a major consideration in prescribing use of these inhibitors.

Chapter 1 : Introduction

1.1. Polo-Like Kinases

1.1.1. Background to Polo-Like Kinases

Polo, a *Drosophila melanogaster* gene product, in which mutations were found to give rise to abnormal spindle poles during mitosis, was first cloned in 1988 (Sunkel and Glover, 1988). Polo kinase is highly conserved from yeast to humans, with eukaryotes having one or more orthologue. CDC5 was recognised as the polo-like kinase (PLK) in *S. cerevisiae* (fission yeast) (Kitada *et al.*, 1993) and Plo1 was discovered in *S. pombe* (budding yeast) (Ohkura, Hagan and Glover, 1995). The PLK family members in *X. laevis* are Plx 1, 2 and 3, with Plx2 and Plx3 being characterised several years after the initial discovery of Plx1 (Kumagai and Dunphy, 1996; Duncan *et al.*, 2001). In mammals, five homologues of polo have been discovered, named PLK1 through to PLK5 (Simmons *et al.*, 1992; Fode *et al.*, 1994; Golsteyn *et al.*, 1994; Donohue *et al.*, 1995; Andrysik *et al.*, 2010) and these proteins are represented in Figure 1.1. The PLKs are a family of serine/threonine protein kinases that have essential roles in several stages of cell cycle progression, through cooperation with Cyclin-dependent kinases (CDKs) (Strebhardt and Ullrich, 2006). They are characterised by a conserved N-terminal serine/threonine kinase domain (with the exception of PLK5) and C-terminal polo box domain, which consists of two polo boxes in the case of PLK1, PLK2, PLK3 and PLK5 or a cryptic polo box domain as seen in PLK4 (Strebhardt, 2010; Slevin *et al.*, 2012). PLK1 is the most highly conserved and best characterised of the PLKs, with essential roles in mitosis, and is the main focus of this thesis. However, a brief overview of the roles of the other four PLKs is given below, before moving on to a more in depth discussion of PLK1.

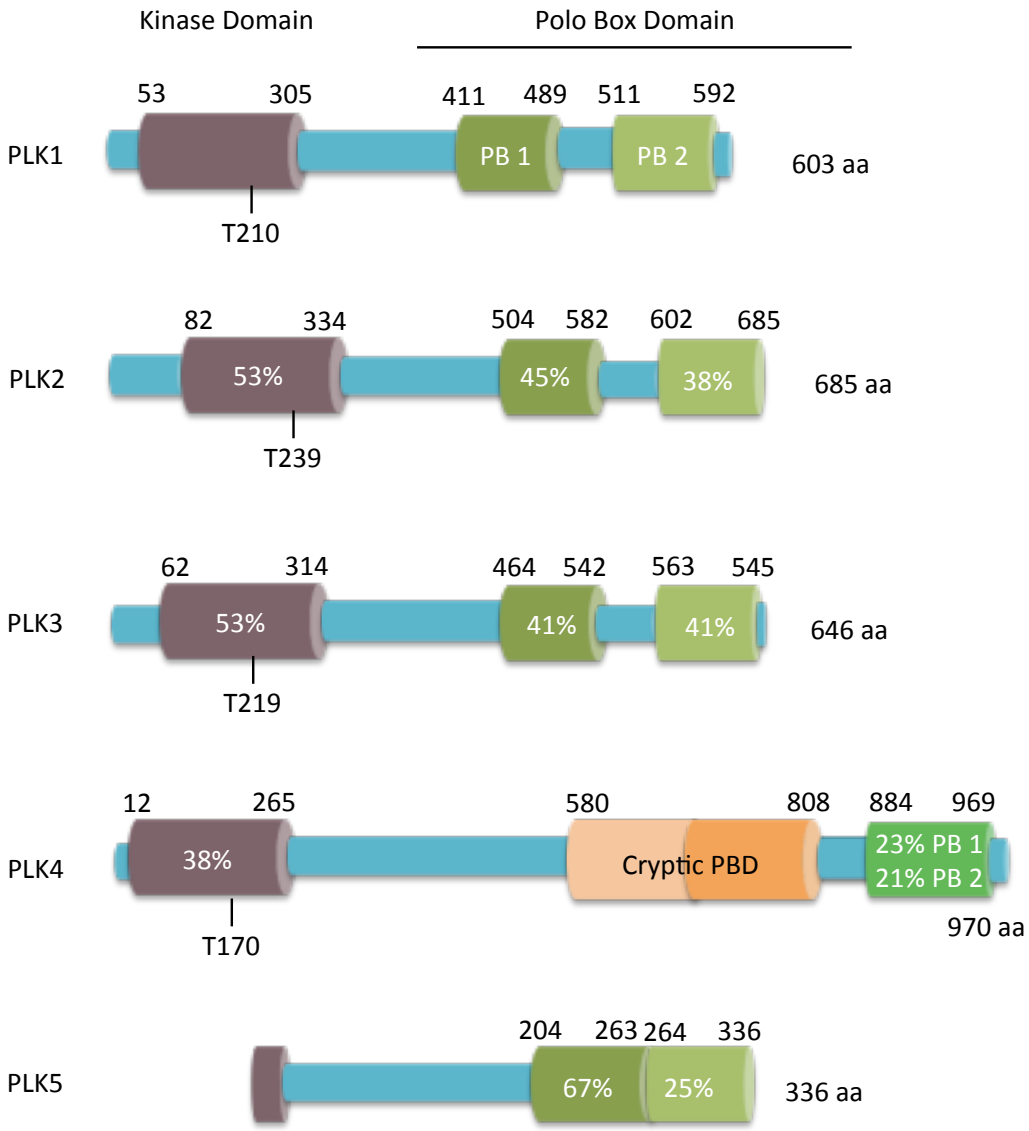


Figure 1.1. The human PLK family. The five members of the PLK family are represented in the schematic. As can be seen, PLK1, PLK2, PLK3 and PLK4 all possess a protein kinase domain (purple) in the N-terminus. The amino acid sequence identity of the kinase domain is represented as a percentage of similarity to PLK1. The activity of PLK1 is enhanced by phosphorylation of threonine 210 (T210) in the activating T-loop in the kinase domain. The threonine residues equivalent to threonine 210 are conserved in PLK2, PLK3 and PLK4, as indicated in the figure. Due to an in-frame stop codon, PLK5 only contains the final fragment of the kinase domain. All five of the PLKs contain a polo box domain (PBD) which comprises two polo boxes (PB 1 and PB 2) (represented in green) in PLK1, PLK2, PLK3 and PLK5. PLK4 contains a cryptic polo box (orange) as well as C-terminal polo box (green). Again, as with the kinase domain, the percentage of sequence similarity of the polo boxes of each PLK is shown in relation to the polo boxes of PLK1. Each protein is a different size, as can be seen from the total number of amino acids (aa) each protein is composed of, which is indicated on the right of each structure. The information in the figure is adapted from (Lee *et al.*, 2014).

1.1.2. Polo-like Kinase-2

The *PLK2* gene maps to chromosome 5 at 5q11.2 and encodes the Polo-like kinase-2 (PLK2) protein. Polo-like kinase-2 (also known as SNK (serum–inducible kinase)) is thought to function as a tumour suppressor. PLK2 was reported to be transcriptionally downregulated in B-cell neoplasms. Silencing of PLK2 occurs frequently in Burkitt lymphoma as well as other types of B-cell neoplasms. The silencing was found to be specific to malignant B cells, with ectopic expression of PLK2 in Burkitt lymphoma cell lines resulting in apoptosis, therefore implicating tumour suppressive roles for PLK2 (Syed *et al.*, 2006). PLK2 is located at the centrosomes during the G1 and S phases of the cell cycle and has been shown to be required for centriole duplication (Warnke *et al.*, 2004; Cizmecioglu *et al.*, 2008). It has been described as a transcriptional target of p53 and is also thought to be involved in activation of a G2/M checkpoint (Burns *et al.*, 2003).

1.1.3. Polo-like Kinase-3

Polo-like kinase-3 (also known as CNK (cytokine-inducible kinase), PRK (proliferation-related kinase) and FNK) is also thought to have tumour suppressive roles. The *PLK3* gene is located at 1p34.1 and PLK3 was firstly identified as a fibroblast growth factor 1 induced immediate entry gene (Donohue *et al.*, 1995). The cell cycle dependent expression of PLK3 is still unclear, as some studies detect similar levels of PLK3 expression throughout the cell cycle (Holtrich *et al.*, 2000; Bahassi *et al.*, 2002), some see a peak at G1 (Zimmerman and Erikson, 2007), some have found the levels peak at late S/ G2 (Ouyang *et al.*, 1997) and some have observed peak levels in mitosis (Chase *et al.*, 1998). PLK3 has been suggested to function at the G1/S transition (Zimmerman and Erikson, 2007) and in the Golgi fragmentation pathway

(Ruan *et al.*, 2004; Lopez-Sanchez, Sanz-Garcia and Lazo, 2009). Like PLK2, PLK3 is a transcriptional target of p53. When ionising radiation is applied, p53 transactivates PLK3 expression (Jen and Cheung, 2005). In addition to PLK3 being a transcriptional target of p53, p53 has been shown to be phosphorylated by PLK3. In response to DNA damage or hydrogen peroxide exposure, PLK3 was found to physically interact with p53 and phosphorylate p53 at serine 20 (Xie, Wang, *et al.*, 2001; Xie, Wu, *et al.*, 2001). Studies have shown that PLK3 expression is negatively associated with certain cancers. Supporting the idea of PLK3 having tumour suppressive roles are the findings that PLK3 mRNA expression is downregulated in lung cancer (Li *et al.*, 1996) and PLK3 null mice are viable, but develop tumours in various organs (Yang *et al.*, 2008). PLK3 was also found to be downregulated in osteosarcomas, with increased expression of PLK3 leading to inhibition of cell proliferation and tumorigenesis, and association with improved patient survival (Lv *et al.*, 2015). In addition to roles in cancer, PLK3 has also been linked to roles in prion disease. PLK3 was shown to assist in the clearance of the pathogenic PrP(Sc) protein that occurs in prion disease, and this study suggested that recovering PLK3 at early stages of prion infection may be a potential treatment to prevent accumulation of this infectious protein in the brain (Wang *et al.*, 2015).

1.1.4. Polo-like Kinase-4

Polo-like kinase-4 (also known as SAK (SNK/PLK-akin kinase) and STK18), is structurally the most divergent PLK and is encoded by the *PLK4* gene which maps to chromosome 4 at 4q28.1. Unlike the other PLKs, which contain two polo box domains, PLK4 has two tandem repeat cryptic polo boxes and also contains a third C-terminal polo box (Slevin *et al.*, 2012). PLK4 has critical roles in centriole duplication (Bettencourt-Dias *et al.*, 2005; Habedanck *et al.*, 2005) and the expression levels of PLK4 are similar to PLK1, with levels increasing gradually from S phase through to

mitosis (Swallow *et al.*, 2005). One study showed that unlike PLK3, which is activated by p53, PLK4 is repressed by p53. PLK4 was shown to be downregulated by wild type p53 in several tumour models, but although three putative p53 binding sites were discovered in the PLK4 promoter, p53 was not observed to bind to these sites, suggesting repression of PLK4 was not through direct binding of p53 to the PLK4 promoter (Li *et al.*, 2005). The role of PLK4 in cancer is still not clear, however PLK4 null mice are not viable and heterozygous mice develop spontaneous tumours, mainly in the liver (Swallow *et al.*, 2005). PLK4 has been implicated as a tumour suppressor in a study where PLK4 expression declined during the progression from non-tumourous liver tissue to hepatocellular carcinoma (Pellegrino *et al.*, 2010). However, other studies have found that PLK4 expression is linked to cancer progression. In breast cancer PLK4 was found to be expressed at a higher level in breast cancer tissue compared to normal breast tissue and high levels of PLK4 was shown to be a detrimental prognostic factor for overall survival (Zhenhua Li *et al.*, 2016). In addition, overexpression of PLK4 in *Drosophila* neuroblasts can promote tumorigenesis (Basto *et al.*, 2008).

1.1.5. Polo-like Kinase-5

Polo-like kinase-5 (PLK5) is the least researched of the PLK family. The *PLK5* gene maps to 19p13.3. PLK5 is more similar to PLK2 and PLK3 than PLK1 or PLK4, based on DNA and protein level sequence similarities. However, PLK5 lacks a functional kinase domain. An in-frame stop codon in exon 6 disrupts the kinase domain of PLK5. However, a conserved ATG in the boundary of exons 6 and 7 leads to expression of a short protein that contains the final fragment of the kinase domain, the linker region and two polo-boxes. It is expressed in the eye, brain and ovary of the mouse (de Cárcer *et al.*, 2011). PLK5 is activated in response to DNA damage and has three p53 binding motifs in the promoter region (Andrysik *et al.*, 2010). It has been suggested that PLK5

is a stress-induced protein, responding to a number of stress signals, such as DNA damaging or microtubule disrupting agents. Like PLK3, overexpressing PLK5 results in a G1 cell cycle arrest and apoptosis (Andrysik *et al.*, 2010). Interestingly, PLK5 has been implicated in the formation of neurite processes, and in many brain tumours PLK5 is found to be downregulated. Reintroduction of PLK5 in glioblastoma multiforme cells causes cell death, suggesting a tumour suppressive role for PLK5 (de Cárcer *et al.*, 2011). More recent studies have associated a three nucleotide deletion near the C-terminus of PLK5 with lymphatic metastasis in clear cell renal cell carcinoma. This suggests PLK5 deletion status could be used as an early biomarker for clear cell renal cell carcinoma metastasis (Zhongjun Li *et al.*, 2016).

1.2. Polo-like Kinase-1

PLK1, the founding member and best characterised of the PLK family was first described in 1994 (Golsteyn *et al.*, 1994). The sections below give an overview of PLK1 including its structure, function, regulation, role in cancer and inhibitors being developed to target its activity.

1.2.1. PLK1 Structure

In 1994 a human protein kinase with a high sequence similarity to polo (*Drosophila*) and CDC5 (*S. cerevisiae*) was characterised (Golsteyn *et al.*, 1994). This protein kinase was referred to as PLK1. The *PLK1* gene is located at 16p12.2 (Xu *et al.*, 2013) and encodes the PLK1 protein, which is 603 amino acids in length and has a molecular mass of approximately 68kDa. As with other members of the PLK family, PLK1 has an N-terminal catalytic domain and C-terminal polo box domain (PBD), comprising two polo boxes. PLK1 localises to numerous structures involved in mitosis, such as kinetochores,

centrosomes and the midbody, and this localisation requires the PBD (Elia, Cantley and Yaffe, 2003). Phosphorylation of PLK1 at threonine 210 by Aurora A leads to enhancement of its catalytic activity (Macůrek *et al.*, 2008; Seki *et al.*, 2008). Once PLK1 is activated the PBD specifically recognises phospho-serine/ threonine containing peptides. PLK1 binds to proteins that are already phosphorylated on this specific motif, allowing subsequent phosphorylation of the target protein at other sites (Elia, Cantley and Yaffe, 2003). Some of the PLK1 target substrates are described in Table 1.1. The PBD is therefore essential for the specificity of the kinase domain (Lee *et al.*, 1998; Y.-J. Jang *et al.*, 2002).

Binding of PLK1 to a phospho-epitope shows that priming phosphorylation of a PLK1 target is a crucial step in the localisation and functions of PLK1. It has been shown that this phosphorylated motif can be generated through two mechanisms, either self-priming by PLK1 or a pro-directed kinase can be the priming factor (Lee *et al.*, 2008). A suggestion that PLK1 may be able to carry out self-priming of targets was due to the fact that inhibition of the catalytic activity of PLK1 resulted in a loss of localisation of PLK1 to centrosomes, kinetochores and midbodies. This showed that the catalytic activity of PLK1 was at least in part required for its localisation (Lénárt *et al.*, 2007). PLK1 was also shown to phosphorylate PBIP1, a PLK1 kinetochore binding protein, at the threonine 78 residue. After this initial phosphorylation, PLK1 bound to this phosphorylated threonine motif, ultimately resulting in self recruitment to the kinetochore (Kang *et al.*, 2006). Perhaps more commonly, a pro-directed kinase will generate the phosphorylated epitope required for PLK1 recruitment and protein interaction. Identification of the pro-directed kinases involved in generating this phospho-epitope has been attempted, with CDK1 being implicated as one of the main pro-directed kinases, and MAP kinases potentially being involved, although less frequently (Lowery, Lim and Yaffe, 2005). CDK1 is crucial for the interaction of PLK1

Substrate	Function
PBIP1	Centromere component important for proper chromosome segregation
FoxM1	Transcription factor regulating a number of G2/M specific genes
CDC25C	Phosphatase that triggers entry into mitosis by removing the inhibitory phosphorylation on Cyclin B/CDK1
Bub1	Protein kinase with essential roles in the spindle assembly checkpoint
BubR1	Protein kinase associated with unattached kinetochores and with roles in stabilising kinetochore-microtubule attachments
PICH	DNA helicase that is an essential component of the spindle assembly checkpoint
Wee1	Negative regulator of entry into mitosis by inhibiting CDK1
Myt1	Negative regulator of entry into mitosis by phosphorylating and inactivating CDK1
Cyclin B1	Regulatory protein involved in mitosis. Forms a complex with CDK1 which is then involved in early events of mitosis, such as chromosome condensation, nuclear envelope breakdown and spindle pole assembly
Pericentrin	Centrosomal protein involved in recruitment of proteins to the pericentriolar matrix to ensure proper centrosome and mitotic spindle formation
Nlp	Centrosomal component involved in regulation of centrosome maturation
MST2	Protein kinase with roles in centrosome disjunction
Nek9	Protein kinase with roles in centrosome separation
Ect2	Guanine nucleotide exchange factor required for cytokinesis
Hbo1	Histone acetylase required for DNA replication licensing
Orc2	Component of the origin recognition complex which is required for the initiation of DNA replication
APC/C	E3 ubiquitin ligase involved in mitotic transition from metaphase to anaphase and in promoting mitotic exit and cytokinesis

Table 1.1. List of PLK1 substrates. Some of the PLK1 substrates mentioned in the main text are listed in the table. The functions of these PLK1 targets that are relevant to PLK1 are also described.

with Forkhead box M1 (FoxM1). CDK1 phosphorylates two key residues in the carboxyl terminal domain of FoxM1, which leads to PLK1 interaction with this domain (Fu *et al.*, 2008). CDK1 was also shown to phosphorylate hCenexin1 at serine 796 which resulted in recruitment of PLK1 to this phosphorylated motif and interaction between hCenexin1 and the PBD of PLK1 (Soung *et al.*, 2009). Several other PLK1 targets have also been shown to rely on CDK1 priming of the target for PLK1 interaction, such as CDC25 (Elia, Cantley and Yaffe, 2003), Bub1 (Qi, Tang and Yu, 2006) and BubR1 (Elowe *et al.*, 2007). Once the PBD has localised PLK1 to its subcellular target, through either mode of priming, the kinase domain can be activated to allow PLK1 to carry out its function of phosphorylating one of its numerous substrates.

1.2.2. PLK1 Function

PLK1 is a kinase that is essential in cell cycle progression, shown by the evidence that inactivating mutations of orthologues of PLK1 result in lethality. This occurs in *Drosophila*, budding yeast and fission yeast. Additionally, a homozygous null PLK1 mouse is embryonic lethal, failing to proliferate past the eight-cell stage (Lu *et al.*, 2008)**.**

PLK1 is a mitotic kinase involved in mitotic entry, regulation of the maturation of centrosomes, spindle assembly and cytokinesis, as well as having several other functions throughout the cell cycle M phase (Barr, Silljé and Nigg, 2004). These functions are summarised in Figure 1.2. Numerous substrates of PLK1 have been discovered and PLK1 has been shown to localise at many different structures. PLK1 is required for almost every step of mitosis, and its cellular localisation changes accordingly throughout mitosis. During interphase and prophase, PLK1 is located at the

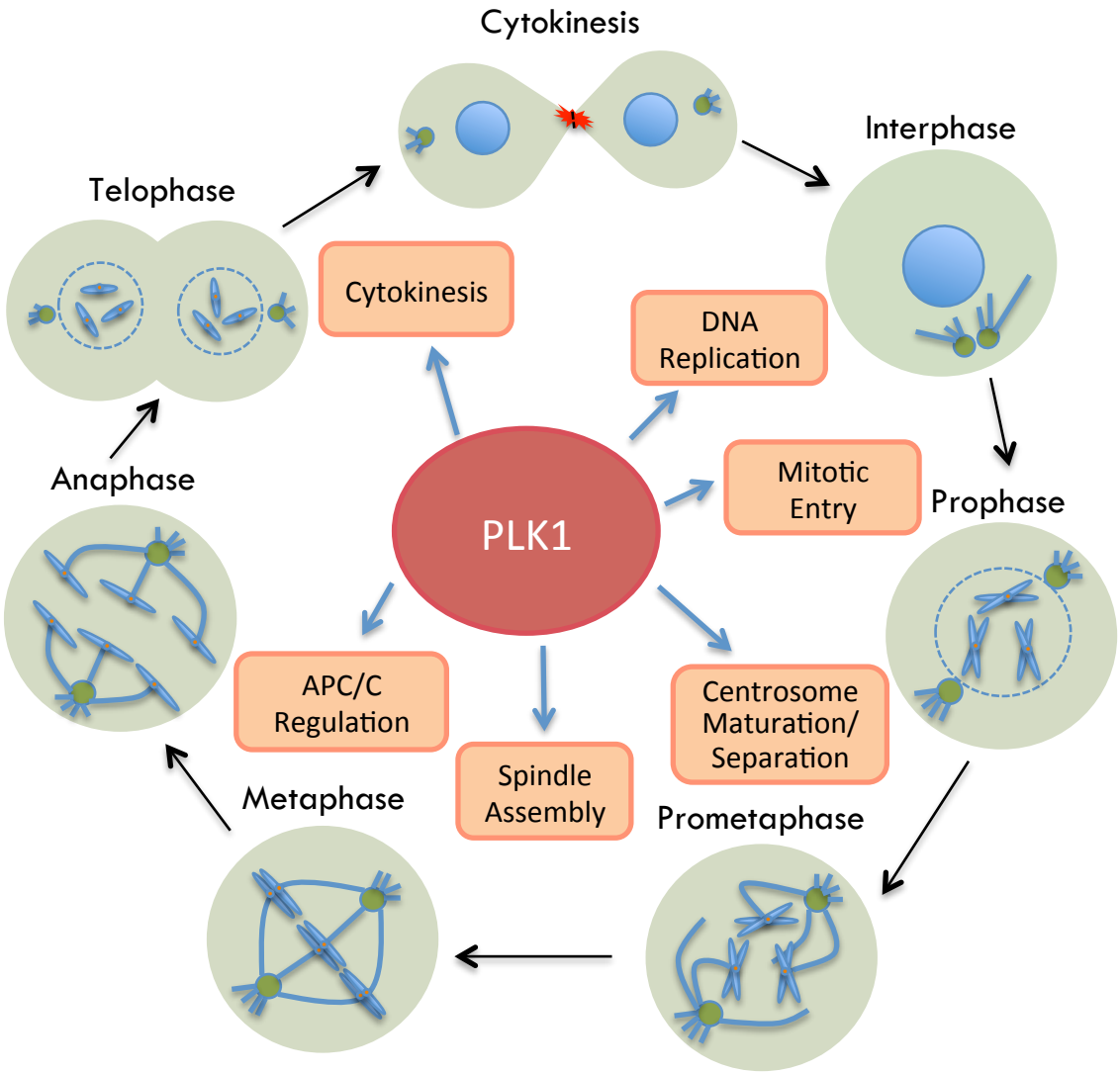


Figure 1.2. The functions of PLK1 during the cell cycle. PLK1 has many roles throughout the cell cycle. The most studied functions of PLK1 are in mitosis, where it has been shown to be involved in several aspects of mitotic progression, such as mitotic entry, centrosome maturation an separation, spindle assembly, APC/C regulation and cytokinesis. PLK1 has also been shown to have roles out with mitosis. In interphase it has been suggested that PLK1 is involved in DNA replication.

centrosomes, then during prometaphase and metaphase it localises to the kinetochores and spindle poles. Throughout anaphase PLK1 localises to the central spindle, and finally in telophase PLK1 is found accumulated on the midbody (Schmucker and Sumara, 2014).

The first major function of PLK1 is in promoting mitotic entry. To achieve this PLK1 phosphorylates several proteins to either result in their activation or promote their degradation, thus leading to activation of the CDK1/Cyclin B1 complex that is essential for mitotic entry. PLK1 phosphorylates CDC25C, an activator of CDK1/Cyclin B1, to assist in the promotion of mitotic entry (Toyoshima-Morimoto, Taniguchi and Nishida, 2002). This activating phosphorylation of CDC25 was initially observed in *Xenopus* with the PLK1 orthologue, Plx1 (Kumagai and Dunphy, 1996). PLK1 also phosphorylates Wee1 and Myt1 leading to their degradation (Nakojima *et al.*, 2003; Van Vugt, Brás and Medema, 2004; Watanabe *et al.*, 2004). This helps promote mitotic entry due to Wee1 and Myt1 having inhibitory functions on CDK1/Cyclin B1. PLK1 also directly phosphorylates Cyclin B1 at the centrosome, which also assists with the entry into mitosis (Toyoshima-Morimoto *et al.*, 2001). Whilst PLK1 has several roles in activating CDK1/Cyclin B1, it does not appear to be essential for mitotic entry, as inhibiting PLK1 leads to a mitotic arrest, therefore suggesting there is not a block in mitotic entry (Steegmaier *et al.*, 2007).

Once entry to mitosis has been achieved, PLK1 is involved in the maturation and separation of centrosomes (Lane and Nigg, 1996). Centrosomes are small organelles that are the microtubule organising centres in mammalian cells (Bornens, 2012). They are composed of a pair of orthogonally arranged centrioles that are surrounded by a mass of dense protein, known as the pericentriolar material (Bettencourt-Dias and Glover, 2007). The pericentriolar material is composed of proteins such as γ-tubulin,

pericentrin and ninein, which are involved in microtubule nucleation and anchoring (Doxsey *et al.*, 1994; Bouckson-Castaing *et al.*, 1996). Centrosomes duplicate once per cell cycle during S phase, a process that requires CDK2 (Matsumoto, Hayashi and Nishida, 1999). Once duplicated, centrosome maturation can occur. Centrosome maturation is the process in which the pericentriolar material structurally changes during mitosis, by increasing the γ-tubulin ring complexes and recruiting extra components (Khodjakov and Rieder, 1999). Centrosome maturation is an important step in the centrosome cycle, as the increase in γ-tubulin results in an improved capacity of the centrosomes to nucleate the microtubules (Moritz *et al.*, 1995; Zheng *et al.*, 1995). The role of PLK1 in this process is thought to be through phosphorylation of targets that lead to recruitment of γ-tubulin and other proteins that are components of the pericentriolar material (Sunkel and Glover, 1988; Lane and Nigg, 1996; Nigg, Blangy and Lane, 1996). PLK1 was shown to phosphorylate pericentrin, which may be an initiating step in centrosome maturation (Lee and Rhee, 2011). Another centrosome associated substrate of PLK1 that has been identified is ninein-like protein (Nlp). Nlp has been shown to interact with the γ-tubulin ring complexes during interphase, and assist in the organisation of microtubules during this stage of the cell cycle. However, in mitosis PLK1 phosphorylates Nlp, leading to its displacement from the centrosome. This allows the establishment of a mitotic scaffold, possibly due to allowing recruitment of further proteins (Casenghi *et al.*, 2003).

After centrosome maturation, the next step in the centrosome cycle is centrosome separation. In human cells the separation of centrosomes allows formation of a bipolar spindle and is an essential step in mitosis to ensure correct chromosome segregation (Meraldi and Nigg, 2002). The process involved in centrosome separation is completed in two steps, and PLK1 has been shown to be involved in both of these steps (Smith *et al.*, 2011) as summarised in Figure 1.3. The first step is centrosome disjunction. During

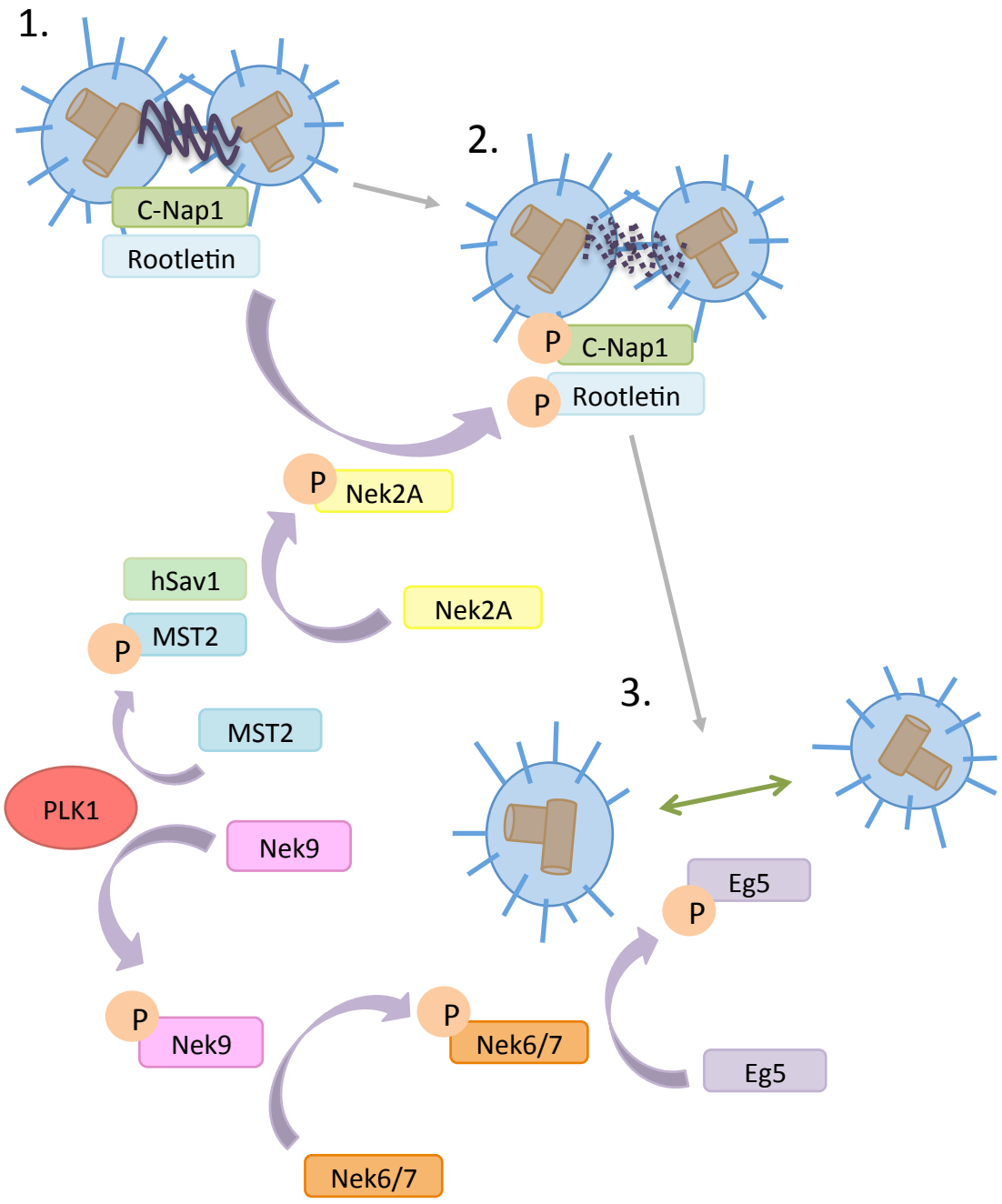


Figure 1.3. The role of PLK1 in centrosome disjunction and separation. PLK1 is involved in both centrosome disjunction and centrosome separation. 1. The centrosomes are joined by the linker of C-Nap1 and rootletin. 2. A phosphorylation cascade leads to centrosome disjunction. PLK1 phosphorylates MST2, which along with hSav1 phosphorylates Nek2A. Nek2A subsequently phosphorylates C-Nap1 and rootletin resulting in the removal of the linker. 3. Centrosome separation is powered by the motor protein Eg5. PLK1 phosphorylates Nek9, which subsequently phosphorylates Nek6/7, finally resulting in phosphorylation and activation of Eg5. Eg5 can then physically drive the centrosomes apart.

interphase, the centrosomes are held together by a linker consisting of the proteins C-Nap1 and rootletin (Fry *et al.*, 1998; Mayor *et al.*, 2000; Bahe *et al.*, 2005). However, once entry into mitosis is achieved, phosphorylation of the linker proteins results in their displacement from the centrosomes allowing centrosome disjunction to occur. The phosphorylation of C-Nap1 and rootletin is part of a phosphorylation cascade. Nek2A phosphorylates C-Nap1 and rootletin (Faragher and Fry, 2003; Bahe *et al.*, 2005), however, Nek2A is regulated by MST2 and hSav1, components of the Hippo pathway (Mardin *et al.*, 2010). PLK1 was found to phosphorylate MST2, and is therefore an upstream activator of centrosome disjunction (Mardin *et al.*, 2011). In parallel to the Nek2A pathway, the second step of centrosome separation involves the motor protein Eg5 driving the centrosomes apart. Eg5 (also known as kinesin-5 and Kif11) is a microtubule-dependent kinesin-like protein, that hydrolyses ATP and converts chemical energy into mechanical energy (Zima, 1998). Eg5 is important in generating a bipolar spindle and as a result has become a cancer therapeutic target (Sawin *et al.*, 1992; Duhl and Renhowe, 2005). PLK1 was shown to be required for Eg5 localisation on the centrosome (Smith *et al.*, 2011). Furthermore, Eg5 activity requires phosphorylation. CDK1 has been shown to phosphorylate threonine 926 of Eg5 to result in its activation (Nigg, Blangy and Lane, 1996; Cahu *et al.*, 2008). However, Nek6/7 also phosphorylate Eg5 at another site, serine 1033 (Rapley *et al.*, 2008). Activation of Nek6/7 requires phosphorylation by Nek9, which has been shown to be activated by PLK1 (Bertran *et al.*, 2011). Overall, PLK1 plays several roles in the centrosome cycle, from maturation to both steps of centrosome separation.

As mentioned, PLK1 also localises at the kinetochores during mitosis. The kinetochores are the chromosomal structures that the microtubules attach to, and due to this localisation of PLK1 it seemed likely that PLK1 may function at the kinetochore (Arnaud, Pines and Nigg, 1998). Subsequent studies showed PLK1 was involved in

microtubule-kinetochore attachments, with depletion of PLK1 leading to a reduction in the number of microtubule-kinetochore attachments (Sumara *et al.*, 2004; Lénárt *et al.*, 2007). One substrate of PLK1 that has been suggested to recruit PLK1 to the kinetochore is PBIP1 (Kang *et al.*, 2006). Several others have now also been described including Bub1 (Qi, Tang and Yu, 2006) and the outer kinetochore protein NudC (Nishino *et al.*, 2006). The functions of PLK1 once at the kinetochore seem to be in regards to the spindle assembly checkpoint. Kinetochores that activate the spindle assembly checkpoint (kinetochores on chromosomes that are not under tension due to incomplete attachments to the microtubules) have high levels of PLK1 (Lénárt *et al.*, 2007). PLK1 has been shown to phosphorylate proteins that are required for the spindle assembly checkpoint, such as Bub1 (Qi, Tang and Yu, 2006), BubR1 (Suijkerbuijk *et al.*, 2012) and PICH (Baumann *et al.*, 2007). However, inactivation of PLK1 does not prevent the spindle assembly checkpoint from occurring, showing that the phosphorylation of the proteins required for the spindle assembly checkpoint by PLK1 is not an essential step for this checkpoint (Lénárt *et al.*, 2007). The localisation of PLK1 at the kinetochores may therefore be for functions in maintaining the microtubule-kinetochore attachments. Supporting this idea is the fact that the PLK1 target, BubR1, is not only required for the spindle assembly checkpoint, but also microtubule-kinetochore attachments (Lampson and Kapoor, 2005).

The functions of PLK1 have been greatly studied in early mitosis. However, PLK1 has also been implicated in later stages of mitosis, having roles in anaphase promoting complex/cyclosome (APC/C) regulation and cytokinesis. APC/C coordinates sister chromatid separation and mitotic exit. In the absence of PLK1 function onset of anaphase and mitotic exit is impaired, suggesting that PLK1 contributes to APC/C regulation (Barr, Silljé and Nigg, 2004). PLK1 has also been suggested to initiate cytokinesis by promoting recruitment of the RhoGEF Ect2 to the central spindle.

Localisation of Ect2 to the central spindle during anaphase is required to stimulate the activation of RhoA GTPase. RhoA GTPase can then assemble and contract the contractile ring, to generate a cleavage furrow, which is a requirement of separating the two daughter cells (Petronczki *et al.*, 2007).

Out with mitosis, PLK1 has more recently been implicated to have functions in DNA replication. An RNAi approach showed that PLK1 is required for DNA replication during cell cycle progression as PLK1 depletion resulted in disruption of the pre-replicative complex formation (Yim and Erikson, 2009). The pre-replicative complex is a protein complex that forms at the origin of replication during DNA replication initiation. PLK1 was shown to phosphorylate the origin recognition complex 1 (Hbo1), which was suggested to be an event required for formation of the pre-replicative complex (Wu and Liu, 2008). A further study showed that Orc2 (origin recognition complex 2), which is a component of the DNA replication machinery, is a target of PLK1 under stressed conditions. PLK1 was shown to phosphorylate Orc2 at serine 188, with enhanced phosphorylation occurring when DNA replication was under challenge (Song *et al.*, 2011). Additionally, FOR20 (FOP-related protein of 20kDa) was shown to be essential for S phase progression and recruitment of PLK1 to the centrosomes. The recruitment of PLK1 to the centrosomes was shown to be a crucial step in DNA replication. This recruitment by FOR20 may therefore be an intra-S-phase signal that maintains proper S phase progression (Shen *et al.*, 2013).

1.2.3. Regulation of PLK1

The levels of PLK1 are required to be tightly regulated during the cell cycle, as expression of hyperactive PLK1 can lead to the G2 checkpoint (which is initiated via DNA damage and results in G2 arrest) being overridden (Strebhardt and Ullrich, 2006).

The activity of PLK1 is regulated in many ways. Golsteyn et al. (1994) showed that the levels of PLK1 are low through the G1 and S phases, begin to increase towards the end of G2, and finally peak during M phase. In addition, actively proliferating tissues have increased levels of PLK1 compared to non-dividing cells (Winkles and Alberts, 2005).

In order for PLK1 to carry out its functions it requires activation. Phosphorylation of PLK1 at the conserved threonine residue, 210, located in the T-loop of the kinase domain is critical for PLK1 activation (Y. J. Jang *et al.*, 2002). During the G2 phase of the cell cycle CDK1 promotes interaction of BORA (an Aurora A co-factor) with the kinase domain of PLK1. This causes a conformational change to PLK1 which allows Aurora A to phosphorylate PLK1 at threonine 210 resulting in its activation (Hutterer *et al.*, 2006; Macůrek *et al.*, 2008; Seki *et al.*, 2008).

At the end of mitosis the PLK1 activity and levels decrease. Upon exit from mitosis the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, targets PLK1 for degradation by the proteasome (Ferris, Maloid and Li, 1998; Lindon and Pines, 2004). This shows that PLK1 expression is regulated throughout the cell cycle, in a cell cycle dependent manner. The factor that controls the cell cycle regulation of PLK1, by repressing PLK1 transcription, is the CDE/CHR (cell-cycle dependent element/cell cycle gene homology region) element found in the PLK1 promoter (Müller and Engeland, 2010). Mutations in this element resulted in a loss of cell cycle specific regulation of PLK1 (Uchiumi, Longo and Ferris, 1997). The CDE was first seen in the CDC25C promoter (Lucibello *et al.*, 1995). CDC25C, is a cell cycle regulated G2 and M phase protein, like PLK1. Further studies then identified that Cyclin A and CDK1 also contained this CDE repressor element, in addition to a CHR region (Zwicker *et al.*, 1995).

PLK1 expression is also repressed by p53, following DNA damage. It has been observed that increased levels of p53, caused by treatment with DNA damage inducing agents, leads to decreased levels of PLK1 (McKenzie *et al.*, 2010). The inhibition of transcription of PLK1 that results in decreased PLK1 levels can occur via direct binding of p53 to the PLK1 promoter. This occurs at two p53 response elements, located at -2067 to -2016 (p53 responsive element 1) and -816 to -785 (p53 responsive element 2) (McKenzie *et al.*, 2010). In addition to a direct p53-dependent repression of PLK1, p53 also activates its downstream target, p21, which can then bind to the promoter region of PLK1, also inhibiting its transcription (Lin *et al.*, 2014). The dimerization partner, RB-like, E2F and MuvB (DREAM) complex can also repress PLK1 expression by binding to the CDE and CHR cell cycle promoter elements. This mechanism of repression is dependent on p21 activation by p53 (Fischer *et al.*, 2015).

1.2.4. PLK1 in the DNA Damage Response

There are many sources of DNA damage, both exogenous and endogenous. When a cell undergoes DNA damage during interphase, particularly the S or G2 phase of the cell cycle, the G2 checkpoint is activated, leading to cell cycle arrest. In this instance PLK1 is not activated. This occurs due to phosphorylation of Bora at threonine 501 by ATM/ATR promoting its degradation by SCF-b-TRCP, an E3 ubiquitin ligase (Qin *et al.*, 2013). Without Bora, PLK1 cannot be activated by Aurora A.

In order for cell cycle progression after restoration of DNA damage, PLK1 activation is required to recover from the checkpoint arrest (Macůrek *et al.*, 2008). Activated PLK1 leads to the subsequent activation of CDC25 which eventually results in mitotic entry. Claspin, an ATR adaptor protein, is also phosphorylated by PLK1, promoting Claspin

degradation. This disturbs the complex of Claspin and ATR, leading to the negative regulation of ATR and thus promoting mitotic entry (Mamely *et al.*, 2006).

1.2.5. PLK1 in Cancer

As PLK1 has been linked to proliferation, it is unsurprising that PLK1 has been associated with cancer, both in terms of cancer development and cancer progression. Elevated PLK1 levels have been shown to be oncogenic and are often correlated with poor prognosis (Wolf *et al.*, 1997; W Weichert *et al.*, 2004). Overexpression of PLK1 is observed in many human cancers, including non-small cell lung cancer (Wolf *et al.*, 1997), head and neck squamous cell carcinoma (Knecht *et al.*, 1999), ovarian cancer (W Weichert *et al.*, 2004), colorectal cancer (Takahashi *et al.*, 2003; Han *et al.*, 2012), breast cancer (Wolf *et al.*, 2000), prostate cancer (Wilko Weichert *et al.*, 2004), gastric cancer (Weichert *et al.*, 2006) and melanoma (Strebhardt *et al.*, 2000), as well as several others (Tut *et al.*, 2015). It was suggested that increased levels of PLK1 could merely be a consequence of increased proliferation in cancer cells and not a driving factor for tumorigenesis. Fitting with this idea was the evidence that the PLK1 gene is rarely mutated. However, NIH3T3, a normal mouse embryonic fibroblast line, were malignantly transformed by transfection and overexpression of PLK1, showing the oncogenic potential of PLK1 (Smith *et al.*, 1997). Interestingly, inhibiting the levels of PLK1 has been reported to reduce the rate of proliferation in cancer cells, without affecting normal cells, therefore it has a great therapeutic potential as a target (Liu, Lei and Erikson, 2006). Whilst overexpression of PLK1 appears to be oncogenic, it is very rare to see mutations in PLK1. Therefore, the up-regulation of PLK1 may be due to upstream proteins involved in its regulation, or epigenetic alterations at the promoter, such as DNA methylation of CpG islands (Weng Ng *et al.*, 2016). In addition to a seemingly oncogenic role, cancers with elevated PLK1 levels also have a poor patient

prognosis and increased risk of metastases (Zhang, Zhang and Kong, 2011; Wang *et al.*, 2012).

1.2.6. PLK1 Inhibition

PLK1 became a target for cancer therapy in light of its oncogenic role. To begin with, different approaches were utilised to determine if inhibiting PLK1 could lead to cancer cell death. Initially, anti-PLK1 antibodies were microinjected into Hela cells, which showed that cellular proliferation was inhibited via an inability of the cells to complete mitosis (Lane and Nigg, 1996). Dominant negative forms of PLK1, single stranded DNA antisense oligonucleotides and siRNA targeting PLK1 were all used to confirm this initial observation (Cogswell *et al.*, 2000; Spänkuch-Schmitt *et al.*, 2002; Liu and Erikson, 2003). In addition to inhibition of cellular proliferation, these studies observed apoptosis of cancer cells, whilst these effects were not observed in normal cells. Due to the promising findings of inhibiting PLK1 it became a valid reason to develop methods of pharmacologically inhibiting PLK1. A number of inhibitors of PLK1 have since been developed with a view of blocking PLK1 in cancer cells that express increased levels of PLK1.

The small molecule inhibitors of PLK1 that are available either target the kinase domain or the PBD. Interestingly, a study comparing the effects of PBD inhibitors with ATP competitive inhibitors found that PBD inhibitors resulted in increases in the proportion of cells in S and G2/M compared to the control, whereas ATP competitive inhibitors only resulted in an increase in cells in G2/M. The PBD inhibitors also resulted in an increase in p21 levels and an S phase arrest, suggesting that these inhibitors affected interphase cells as well as mitotic cells, whereas the ATP competitive inhibitors activated the BubR1 spindle checkpoint kinase, suggesting a mitotic arrest. Overall, this

suggested a role for the PBD in S phase whilst the kinase domain was mainly involved in mitotic progression (Shin, Woo and Yim, 2015). However, both types of inhibitors have shown apoptotic effects in cancer cells and are in preclinical and clinical development.

The PBD is an attractive alternative target to the kinase domain. As previously mentioned, the PBD has roles in subcellular localisation and target specificity of PLK1. Therefore, inhibitors targeting the PBD would interfere with protein interactions that are dependent on the PBD, rather than with ATP binding, as is seen in targeting the kinase domain (Ravichandran *et al.*, 2011). The first PBD inhibitors described were the natural product, thymoquinone, and a synthetic thymoquinone derivative, Poloxin (Reindl *et al.*, 2008). Other PBD inhibitors of PLK1 have now been discovered and developed, including Poloxin-2 (Scharow *et al.*, 2015), Poloxipan (Reindl *et al.*, 2009) and Purpurogallin (Watanabe *et al.*, 2009). However, the inhibitors used in this thesis are ATP competitive kinase domain targeted inhibitors, so they will be focused on more specifically.

The ATP competitive PLK1 inhibitors targeting the kinase domain have increased specificity to PLK1 over other kinases due to certain structural features of PLK1 (Garuti, Roberti and Bottegoni, 2012). One such feature is the presence of a pocket in the hinge region that is created by the Leu132 residue. In other kinases a tyrosine or phenylalanine is often found in this place, which may prevent competitive inhibition (Ravichandran *et al.*, 2011). Additionally, the ATP pocket comprises a bulky phenylalanine at the top, a small cysteine at the base, and an unusually high concentration of positively charged residues in the solvent exposed region (Garuti, Roberti and Bottegoni, 2012). These features confer specificity for an inhibitor targeting

PLK1. Numerous inhibitors are available, but the two that have been used in this thesis are BI6727 and GSK461364.

BI6727, also known as Volasertib, is a highly potent and selective dihydropteridinone derivative PLK1 inhibitor produced by Boehringer Ingelheim (Rudolph *et al.*, 2009). BI6727 selectively inhibits PLK1, resulting in G2/M arrest followed by apoptosis in cancer cells, whilst in normal cells apoptosis is not observed. This small molecule inhibitor therapy results in cell cycle arrest by competitively binding to the ATP binding pocket of PLK1. The binding occurs through two hydrogen bonds from the interior of the backbone of the amine and carbonyl groups of cysteine 133. This results in BI6727 blocking the ATP binding site, preventing the catalytic activation of PLK1 (Weiß and Efferth, 2012). In initial phase I trials, BI6727 was well tolerated with manageable toxicities and antitumour activity in advanced or metastatic solid tumours (Schöffski *et al.*, 2012). Further phase I monotherapy trials also showed promising results (C.C. Lin *et al.*, 2014; Kobayashi *et al.*, 2015; Nokihara *et al.*, 2015). Phase II trials have had mixed results. In a study trial for the treatment of patients with locally advanced metastatic urothelial cancer, BI6727 was shown to have an acceptable safety profile, however further evaluation as a monotherapy was not warranted due to insufficient antitumour activity (Stadler *et al.*, 2014). A second phase II study investigated the effects of BI6727 or Pemetrexed as monotherapies or in combination for the treatment of non-small-cell lung cancer patients. A lack of efficacy of BI6727 as a monotherapy resulted in stopping recruitment of patients to this experimental arm. The combination of Pemetrexed with BI6727 compared to Pemetrexed alone did not result in a significant increase in toxicity, but also did not improve the efficacy. These findings suggested BI6727 was not suitable for treatment of non-smell-cell lung cancer patients (Ellis *et al.*, 2015).

Whilst these two phase II studies did not show promising results for BI6727, a third phase II trial showed significantly better outcomes. Acute myeloid leukaemia patients were treated with low-dose cytarabine alone or in combination with BI6727. The overall outcomes of this trial showed that the combination treatment increased the response rate and improved overall survival in acute myeloid leukaemia patients compared to low-dose cytarabine monotherapy. This was independent of genetic subgroups and although the combination treatment resulted in an increased frequency of adverse events, the safety profile was considered clinically manageable. This therefore warranted further clinical investigation. (Döhner *et al.*, 2014). Further phase I and phase II trials are currently on going, with BI6727 being assessed as both a single agent and in combination with other agents to treat numerous malignancies.

In 2013 BI6727 received FDA breakthrough therapy designation for treatment of patients with acute myeloid leukaemia. The phase III study investigated BI6727 in combination with low-dose cytarabine in patients over 65 years of age. This makes BI6727 the PLK1 inhibitor that is furthest through the clinical trial pipeline, and the lead agent in the PLK1 inhibition field. In 2016 a statement was released from Boehringer Ingelheim stating that BI6727 had not met the primary endpoint of objective response. Whilst the percentage of patients with objective response was higher in the BI6727 and low-dose cytarabine cohort compared to low-dose cytarabine plus placebo group, the findings were not significant. This was in part due to severe adverse events occurring in the experimental arm (Döhner *et al*., 2016). With many other trials still ongoing, there is still potential for BI6727 in the clinic. Additionally, a recent study suggested that combining a proteasome inhibitor with BI6727 leads to enhanced mitotic arrest in acute myeloid leukaemia *in vitro* and prolonged survival of a xenotransplant mouse model of human acute myeloid leukaemia *in vivo,* suggesting that further

research into suitable combination treatments could enhance the efficacy of BI6727 (Schnerch *et al.*, 2017).

GSK461364 is a small molecule PLK1 inhibitor developed by GlaxoSmithKline. *In vitro* studies have shown that GSK461364 has a higher selectivity for PLK1 compared to PLK2 and PLK3, than BI6727 (Medema, Lin and Yang, 2011). In preclinical studies GSK461364 was found to have increased antiproliferative activity in p53 mutated cancers compared to cancers where wild type p53 is retained (Degenhardt *et al.*, 2010). This could be a very important finding, as many of the current cancer therapies have more successful results in treatment of p53 competent cancers, and p53 is mutated in around 50% of cancers. In phase I clinical trials, the pharmacokinetic and pharmacodynamics profiles of GSK461364 were assessed in patients with advanced solid malignancies. The best response observed was prolonged stable disease for more than 16 weeks (Olmos *et al.*, 2011). No further clinical trial studies have been registered for GSK461364, however there are many research papers published on this drug. A recent study showed that GSK461364 treatment of neuroblastoma cell lines resulted in cell cycle arrest and induced apoptosis. Treatment with GSK461364 also delayed established xenograft tumour growth in nude mice and increased survival time. This suggested that treatment with this drug may benefit neuroblastoma patients with elevated PLK1 levels (Pajtler *et al.*, 2017). In osteosarcoma cells GSK461364 was also shown to result in a G2/M arrest and cytotoxic effect through the induction of apoptosis (Chou *et al.*, 2016). Therefore with future work, GSK461364 may enter further clinical trials.

The results of clinical evaluation of PLK1 inhibitors has shown that while they show promise in the clinic, improvement is required in decreasing the toxic side effects to allow increased doses which may be more successful in targeting cancer cells.

1.2.7. PLK1 and p53

As previously mentioned, p53 has been shown to repress PLK1. However, whilst p53 can repress PLK1, PLK1 has also been reported to repress p53, in a negative feedback loop. PLK1 can repress p53 through direct binding. In mammalian cultured cells, PLK1 was found to physically interact with p53, leading to loss of p53 pro-apoptotic function. However, the biological significance of this is uncertain due to this being based on a single study (Ando *et al.*, 2004). Indirect mechanisms have also been reported to exist. PLK1 was shown to stabilise MDM2, by phosphorylation of serine 260. This promotes MDM2 mediated turnover of p53 (Dias *et al.*, 2009). Therefore, it has been shown that a negative feedback loop exists between p53 and PLK1, with p53 repressing PLK1 transcription and PLK1 inhibiting p53 function. This interaction has been represented as a schematic in Figure 1.4. The interaction between these two proteins is complex, and not fully understood. Numerous studies have been published that research the regulation of each protein by the other, either directly or indirectly. However, there is also a lot of interest in the correlation between treatment with PLK1 inhibitors and the p53 status of cancers. Conflicting reports currently exist in the literature with one study suggesting that p53 does not influence the sensitivity of cancer cells to PLK1 inhibition (Sanhaji *et al.*, 2012), whilst several other studies show that p53 does alter the sensitivity of cancer cells to PLK1 inhibitors (Guan *et al.*, 2005; Liu, Lei and Erikson, 2006; Sur *et al.*, 2009; Degenhardt *et al.*, 2010; McKenzie *et al.*, 2010; Danovi *et al.*, 2013; Yim and Erikson, 2014). To gain a better understanding of p53, an introduction to this highly researched protein follows.

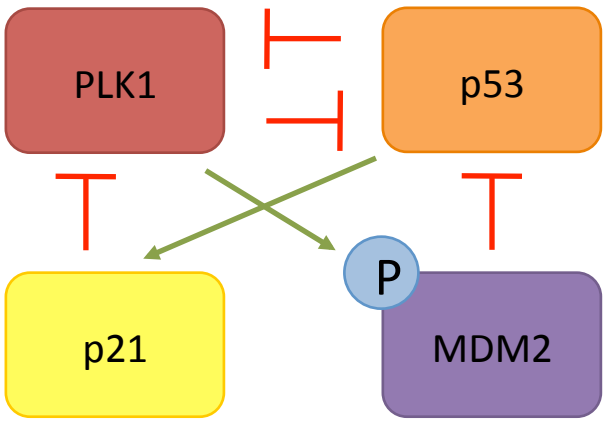


Figure 1.4. Schematic representation of the interaction between p53, PLK1, MDM2 and p21. Studies have reported that p53 and PLK1 are involved in negative regulation of each other. PLK1 was shown to directly inhibit p53 function, as well as promote p53 degradation through phosphorylation of MDM2. However, p53 has been shown to repress PLK1 expression through both directly binding to the PLK1 promoter, and also through activation of p21, which was also shown to inhibit PLK1.

1.3. p53

1.3.1. Background of p53

The discovery of p53 occurred in 1979, with several independent groups of investigators reporting the discovery of an approximately 53kDa protein (Kress *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero *et al.*, 1979; Smith, Smith and Paucha, 1979; Rotter *et al.*, 1980). *TP53*, the gene encoding the p53 protein was initially believed to be an oncogene, due to p53 being discovered as a cellular partner of simian virus 40 (SV40) large T-antigen, which is a known oncogene product. Observations were also made that transformed cell lines and cells from patient's tumours had elevated p53 levels and p53 was located in the nucleus in transformed fibroblasts, but located in the cytoplasm of non-transformed fibroblasts (DeLeo *et al.*, 1979; Rotter, Abutbul and Ben-Ze'ev, 1983). The first decade of research on p53 supported the idea that p53 was an oncogene. Several labs cloned p53 and observed that transfecting cells with these clones resulted in transformation. It wasn't until realisation that none of the p53 clones being used had the same DNA sequence, that the role of p53 as an oncogene was questioned. It became apparent that whilst mutated p53 could transform cells, wild type p53 could not (Finlay, Hinds and Levine, 1989). This then began the era of research of p53 as a tumour suppressor.

Early studies showed that p53 was mutated in several cancers, and now it is known that p53 is mutated in approximately 50% of all cancers (Vogelstein, Lane and Levine, 2000). The main focus of p53 research for many years has been aimed at investigating the role of p53 as a tumour suppressor in cancer, due to this p53 was termed the 'guardian of the genome' (Lane, 1992). In the past 38 years, p53 has been extensively studied and there is currently more than 86000 publications centred on p53. Although

p53 has been shown to be highly important in cancer, there is still no precise answer as to how to use this information to treat cancer patients effectively.

1.3.2. Structure of p53

The p53 protein is encoded by the *TP53* gene, located on the short arm of chromosome 17 at position 13.1, and contains 11 exons and 10 introns (Isobe *et al.*, 1986). The protein was named on the basis of what was thought to be its molecular mass, as it runs at 53 kDa on SDS-PAGE. However, a c-DNA clone of p53 revealed that the protein consists of 393 amino acids and has a molecular weight of 43.5 kDa (Harlow *et al.*, 1985). There are three major functional domains of the protein, the N-terminal activation domain (residues 1-93), the DNA binding (core) domain (residues 94-312), and the C-terminal regulatory domain (residues 325-393) as shown in Figure 1.5. Within the N-terminal domain there are two transcriptional activation (transactivation (TAD)) domains and a proline-rich region. The transactivation domain is essential for functionality of p53 as a transcription factor (Fields and Jang, 1990; Chang *et al.*, 1995) and the proline-rich domain contributes to repression, apoptosis and response to ionising irradiation (Walker and Levine, 1996; Venot *et al.*, 1998; Campbell *et al.*, 2012). The DNA binding core domain is required for sequence specific binding of p53 to DNA (Kern *et al.*, 1991). The most common mutations in p53 occur in this domain and are generally missense mutations. Studies have identified six hotspot mutations in the DNA binding domain, residues R175, G245, R248, R249, R273 and R282. These mutations result in the loss of function of wild type p53, however, they can also lead to gain of novel functions in promoting tumour development (Rivlin *et al.*, 2011). The C-terminal regulatory domain contains sequences for smaller domains and elements, including the nuclear localisation signal, tetramerisation domain, nuclear export signal and the regulatory domain. The tetramerisation domain is required for formation of

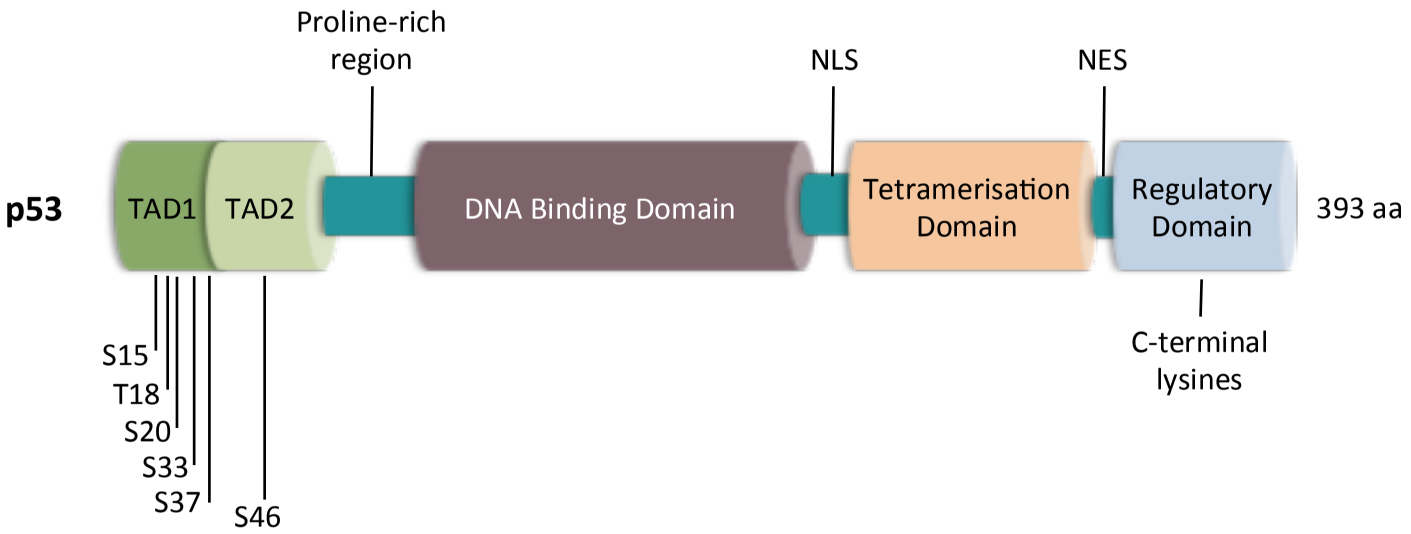


Figure 1.5. p53 structure. A schematic representation of the p53 protein is shown, highlighting important functional domains. TAD1 and TAD2 are the transcriptional activation domains of p53 located at the N-terminus. The NLS is the nuclear localisation sequence and NES is the nuclear export sequence. Some of the N-terminal residues (mentioned in the main text) that commonly undergo phosphorylation in order to regulate p53 in response to DNA damage and other types of stress are indicated. The C-terminal lysines that are found in the regulatory domain and are sites of post-translational modifications are also highlighted.

homo- and hetero-tetramers, which are essential for p53 functions in DNA binding, protein-protein interactions, transactivation and tumour suppression, as well as p53 degradation (Chène, 2001). The nuclear export signal has roles in p53 degradation, and the nuclear localisation signal is important for p53 localisation into the nucleus. Finally, the regulatory domain is regulated by many various post-translational modifications including acetylation by p300/CBP and ubiquitylation by MDM2 (Gu and Roeder, 1997; Rodriguez *et al.*, 2000).

1.3.3. Function of p53

The p53 protein was shown to function as a transcription factor (Raycroft, Wu and Lozano, 1990). Numerous further studies have shown that p53 can regulate the transcription of a large range of target genes, through sequence specific binding to gene promoters containing p53 responsive elements (Kern *et al.*, 1991). The target genes that p53 regulates are involved in a wide range of functions which can be grouped into three categories: survival, through effects such as cell cycle arrest, senescence, DNA repair, autophagy and genome stability; death through apoptosis; and homeostasis, where basal levels of p53 are thought to have roles in fertility, metabolism, stem cell differentiation and immunity (Meek, 2015). Some of these functions of p53 are represented in Figure 1.6.

The basal levels of p53 are low, and under unstressed conditions, p53 is a relatively unstable protein. However, p53 is activated and stabilised in response to a variety of cellular stresses, such as DNA damage, oncogene activation, hypoxia, telomere erosion and viral infection. Other sources of stress that activate p53 are represented in Figure 1.6. Once p53 is activated and the protein levels have stabilised, downstream target genes can be stimulated or repressed, dependent on the stress encountered.

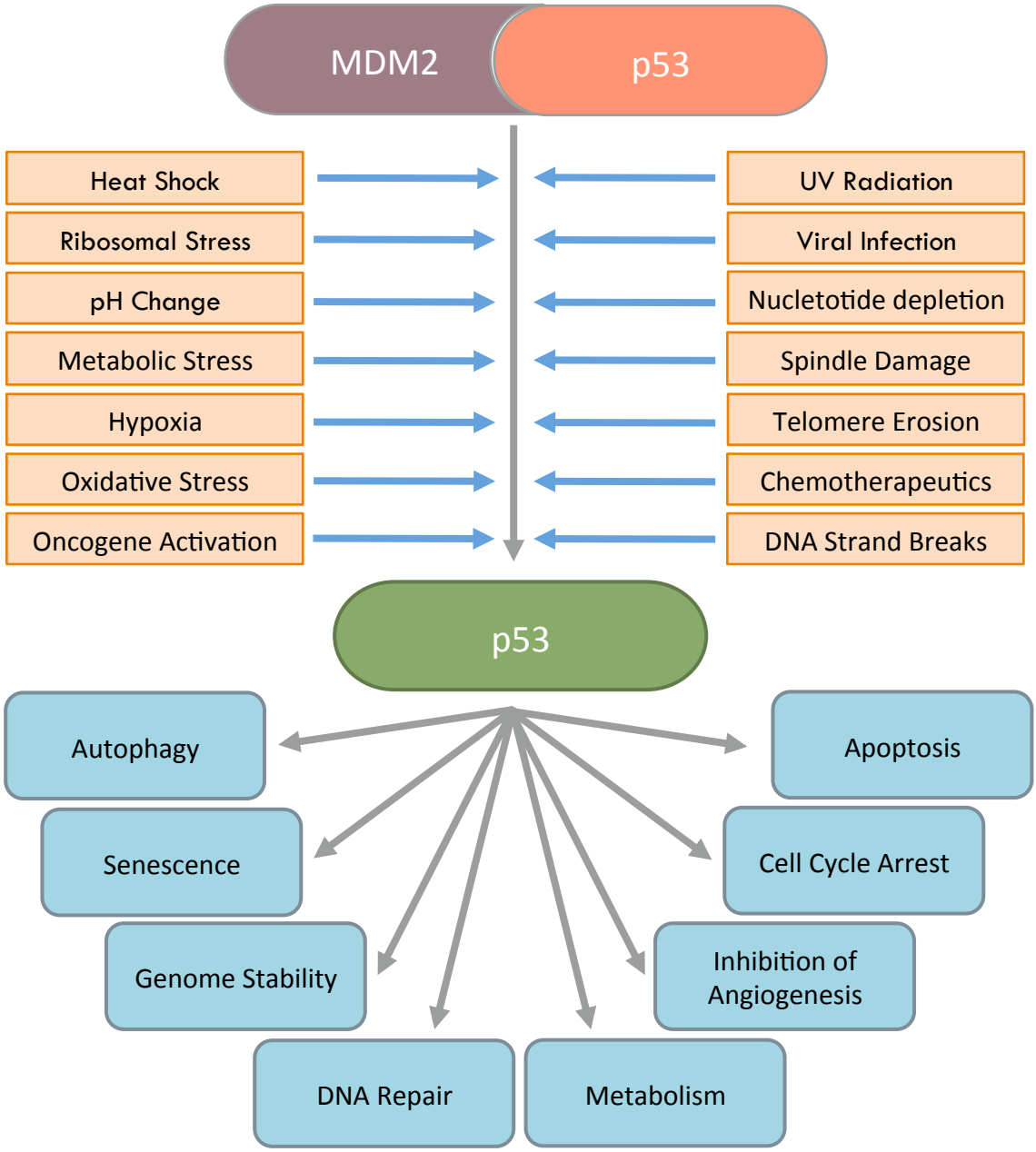


Figure 1.6. The regulation and functions of p53. Under unstressed conditions, p53 is found at low levels in an unstable state. This is in part due to MDM2 mediated degradation. However, upon numerous types of cellular stress, with examples found in the orange boxes, the interaction of p53 and MDM2 is disrupted, allowing stabilisation of p53. Once stabilised, p53 can carry out one of its many functions, which involve activation or repression of genes that are involved in responses such as those indicated in the blue boxes.

p53 is regarded as a tumour suppressor, and so its overall function is to prevent the growth and survival of cancerous cells. Li-Fraumeni syndrome shows the importance of p53 as a tumour suppressor as this syndrome presents with the development of multiple tumours at a young age, and is as a result of an inherited germline mutation of p53 (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Further evidence of the tumour suppressor function of p53 is shown by the fact that although p53 null mice develop normally, they are highly susceptible to tumour development within 6 months after birth (Donehower *et al.*, 1992).

p53 can result in several diverse outcomes for cellular fate. Regulation of the cell cycle is one of the most important functions of p53. The cell cycle is regulated by Cyclin Dependent Kinases and Cyclins. Activation of p53 can result in cells arresting at several different cell cycle checkpoints. To halt the cell cycle, p53 can activate genes that are cell cycle inhibitors. For example p53 can transactivate p21, a cyclin-dependent kinase inhibitor, which results in cell cycle arrest at the G1/S and G2/M boundaries (El-Deiry *et al.*, 1993). Reprimo is another gene stimulated by p53 that inhibits the transition from G2 to M (Ohki *et al.*, 2000). Similarly, 14-3-3σ expression is activated by p53, and results in a G2 arrest by constraining CDK1 and Cyclin B1 complexes out with the nucleus, preventing mitotic entry (Chan *et al.*, 1999; Laronga *et al.*, 2000). The cell cycle arrest imposed by p53 can be temporary to allow repair to occur or can be permanent resulting in total growth arrest, also known as senescence (Shay, Pereira-Smith and Wright, 1991). As well as being able to arrest the cell cycle, p53 can also promote apoptosis, if the stress or damage is severe and irreparable. The decision to undergo apoptosis is also dependent on the cell type, duration of stress and the type and level of stress encountered. To do this p53 upregulates genes that are pro-apoptotic (e.g BH3 family genes) and downregulates genes that are pro-survival (e.g Bcl-2 family genes), to tip the balance towards cell death rather than cell survival (Selvakumaran *et*

al., 1994). Studies have shown that p53 can stimulate the expression of numerous pro-apoptotic proteins, such as BAX (Toshiyuki and Reed, 1995) and PUMA (Nakano and Vousden, 2001).

Prevention of genomic instability is another major function of p53. There is evidence to suggest that p53 has roles at the centrosome. Studies have shown that cells lacking p53 have multiple centrosomes, therefore implicating p53 as having roles in regulating centrosome duplication (Fukasawa *et al.*, 1996). If centrosome hyperamplification occurs, it can lead to genomic instability as multiple poles can arise, preventing the formation of a normal bipolar spindle, which can permit aneuploidy. This effect has also been shown *in vivo*, as a correlation between p53-null mice and centrosome hyperamplification and aneuploidy was observed (Fukasawa *et al.*, 1997). Clinical implications were also observed as loss of p53 function was associated with increased centrosome numbers in breast and head and neck cancers (Carroll *et al.*, 1999). Not only does p53 play a role in preventing centrosome hyperamplification, it also acts to prevent cells continuing through the cell cycle when centriole duplication fails. A recent study showed that depletion of PLK4, which leads to centriole duplication failure, resulted in an irreversible cell cycle arrest. However, depletion of p53 in this scenario allowed proliferation to continue, with no cell cycle arrest taking place (Lambrus *et al.*, 2015). Another study showed that inhibition of centriole duplication led to a G1/S arrest through p53 upregulation, which was dependent on p38, a kinase induced by cellular stress, rather than ATM, a DNA damage induced kinase (Song *et al.*, 2010). The exact mechanism by which p53 may execute such roles is currently unknown. However, p53 has also been shown to localise to the centrosomes, so direct association with the centrosomes may be an important aspect (Morris *et al.*, 2000). One study showed that the localisation of p53 at the centrosomes was dependent on phosphorylation of p53 at serine 15 by ATM (Tritarelli *et al.*, 2004). As well as roles in regulation of

centriole/centrosome duplication, p53 has also been shown to regulate appropriate timing of centrosome separation. Cyclin B2 was shown to trigger increased activation of Aurora A, which led to upregulation of PLK1. This promoted accelerated centrosome separation. However, p53 acted antagonistically to Cyclin B2, to keep Aurora A mediated activation of PLK1 in check, which prevented the accelerated centrosome separation (Nam and van Deursen, 2014). Clearly there are several potential roles for p53 at the centrosomes, which will hopefully be uncovered and underpinned by future studies.

Several studies have shown that p53 can prevent karyotypic instability by preventing polyploid cells re-entering the cell cycle. Cells that fail to segregate their chromosomes correctly often do not complete cytokinesis, which results in cells entering G1 with 4N DNA. In p53 competent cells, cells that do not contain the normal 2N DNA complement are arrested and fail to continue through the cell cycle. However, cells lacking p53 were seen to re-enter S phase with 4N DNA (Cross *et al.*, 1995). It has been suggested that p53 is only activated in response to tetraploid cells after mitotic exit. The duration of mitotic arrest was indistinguishable between p53 competent and p53 deficient cells suggesting that p53 does not have a direct role in mitosis. Furthermore, p53 competent cells that fail to complete cytokinesis arrest with 4N DNA and lack MPM-2 expression (a mitosis specific protein) but express Cyclin E and unphosphorylated pRb and have uncondensed chromosomes, suggesting they are arrested in a G1 like state (Lanni and Jacks, 1998). Overall, p53 has been implicated to function in a post mitotic checkpoint in response to mitotic failure. It has been suggested that p53 is activated in response to mitotic spindle damage through interaction with BubR1 leading to increased p53 phosphorylation (Ha *et al.*, 2007).

Angiogenesis is an important step in the development of cancer. This is where new blood vessels are formed to feed the cancer cells, to allow the tumour to grow. Growth factors, such as vascular epithelial growth factor (VEGF) and fibroblast growth factor (FGF) have been implicated in this process. However, p53 has been shown to play roles in inhibiting this process, adding another aspect of tumour suppressive roles for p53. To help in the inhibition of angiogenesis p53 stimulates the expression of natural angiogenesis inhibitors, such as alpha collagen (Teodoro *et al.*, 2006) and thrombospondin (Dameron *et al.*, 1994).

Invasion and metastases are another two aspects of tumour development. Invasion occurs when cancer cells invade neighbouring tissues, and metastasis is the spread of cancer cells to another part of the body. The tumour suppressor functions of p53 act to inhibit these processes. The p53 dependent upregulation of genes such as MASPIN, can help to inhibit metastasis (Zou *et al.*, 2000).

Although much of the research on p53 has been focused in the context of cancer, there are a number of other functions for p53 out with this area. Involvement in homeostasis, to maintain normal physiological conditions, is another area that p53 has been implicated as having a role. Fertility/reproduction can be enhanced in mice by p53 through the upregulation of leukaemia inhibitory factor. This factor is essential for blastocyte implantation (Hu *et al.*, 2007, 2008). Roles for p53 in ageing, metabolism, autophagy, glycolysis, differentiation and miRNA processing have also all been shown (Vousden and Prives, 2009).

Neurodegenerative diseases have also been linked to p53. The apoptotic functions of p53 can lead to neuronal death, thus mediating neurogenerative conditions. In Alzheimer's disease p53 has been implicated to contribute to neuronal death (Culmsee and Landshamer, 2006) and in Parkinson's disease p53 has been shown to lead to

dopaminergic neuron death (Qi *et al.*, 2016). Additionally, Huntington's disease is an inherited disorder that ultimately results in the death of brain cells. Mutant forms of huntingtin that are the cause of Huntington's disease activate p53. In mouse models this activation of p53 was observed to be involved in the neurodegeneration and neurobehavioural abnormalities (Bae *et al.*, 2005). Additionally, p53 was shown to induce the expression of huntingtin which was predicted to further disease progression (Feng *et al.*, 2006).

1.3.4. Regulation of p53

In unstressed conditions, the levels of p53 are relatively low in the cell. This is due negative regulators of p53. One of the best studied and most important regulators of p53 is MDM2 (murine double minute 2). MDM2 was first identified to interact with p53 in 1992 (Barak and Oren, 1992; Momand *et al.*, 1992; Oliner *et al.*, 1992). MDM2 is a RING-finger E3 ubiquitin ligase that contains a p53 binding domain in its N-terminus. This allows specific interaction with p53, resulting in promotion of p53 ubiquitylation by the C-terminal RING-finger motif leading to p53 degradation. However, the RING-finger can also target MDM2 for auto-ubiquitylation and resulting degradation. The negative regulation of p53 by MDM2 occurs in an autoregulatory feedback loop. MDM2 is a transcriptional target of p53, therefore expression of MDM2 can be regulated by the levels of p53, but this results in increased repression of p53 (Wu *et al.*, 1993). Evidence showing the negative regulation of p53 by MDM2 comes from a study that shows MDM2 knockout mice are embryonic lethal, but if p53 is also knocked out then the lethality is rescued (Jones *et al.*, 1995; Montes de Oca Luna, Wagner and Lozano, 1995). The regulation of p53 is tightly controlled by MDM2, and for p53 stabilisation to occur the interaction of MDM2 and p53 has to be disturbed.

Phosphorylation of p53 is a key event in the disruption of the MDM2-p53 complex. Phosphorylation of the serine 15 residue of p53 was reported to result in a conformational change in p53 that reduces the ability of MDM2 to bind to p53 (Shieh *et al.*, 1997). Phosphorylation of serine 20 was also suggested to be an important event in p53 stabilisation. Mutating serine 20 to alanine resulted in a high sensitivity of p53 to MDM2 mediated degradation (Chehab *et al.*, 1999; Dumaz *et al.*, 2001). However, another study suggested that phosphorylation of threonine 18, and not serine 15 or serine 20, was a key event in weakening MDM2 binding affinity for p53 (Schon *et al.*, 2002). Although, phosphorylation of serine 15 enhances subsequent phosphorylation of threonine 18, therefore implicating serine 15 phosphorylation as an important step in the disruption of MDM2 and p53 whether directly or indirectly (Dumaz, Milne and Meek, 1999). Phosphorylation of serine 15 results in stabilisation of p300 and CBP binding to p53 (Lambert *et al.*, 1998; Dumaz and Meek, 1999). Due to p300 and CBP having transcriptional factor acetyl-transferase activity, p53 is subsequently acetylated at C-terminal lysine residues 370, 372, 373, 381 and 382. Some of these sites are targets of MDM2 ubiquitylation, and as a result acetylation of p53 inhibits ubiquitylation of p53 by MDM2 (Li *et al.*, 2002).

Numerous different proteins are involved in the activation of p53 depending on the cellular stress and the cell type. p53 has been shown to undergo many post-translational modifications. It has been shown that the N-terminus of p53 is heavily phosphorylated, whilst several C-terminal lysines (as indicated in Figure 1.5) are sites of phosphorylation, acetylation, methylation, ubiquitylation, sumoylation and neddylation. Several proteins can be responsible for these modifications, such as Chk1, Chk2, ATM, ATR, DNA-PK, p38, HIPK2 and JNK, with some of these examples discussed in more detail below. These proteins modify p53 on different sites, which is thought to indicate the type of stress allowing p53 to carry out the appropriate response.

One of the best studied activators of p53 is DNA damage. In response to single or double strand breaks p53 is phosphorylated at serine 15, principally by ATM or ATR. The phosphorylation of serine 15 is a critical event for transactivation of p53 target genes, such as p21 (Loughery *et al.*, 2014), and also leads to subsequent sequential modifications of p53 (Sakaguchi *et al.*, 1998). Serine 15 and serine 37 were also shown to be phosphorylated by DNA-PK in response to DNA damage (Lees-Miller *et al.*, 1992). Phosphorylation of serine 20 has also been implicated to have an important role in p53 activation. As previously mentioned, the serine 20 residue of p53 is phosphorylated by PLK3 upon exposure to hydrogen peroxide (Xie, Wang, *et al.*, 2001). In addition, whilst ATM and ATR are not directly involved in phosphorylating serine 20, in response to DNA damage, the ATM target Chk2 has been shown to phosphorylate this site (Hirao, 2000).

UV radiation is another known activator of p53. In this instance, p38, a mitogen-activated protein kinase (MAPK) has been shown to phosphorylate p53 leading to its activation. Studies have shown p38 can phosphorylate serine 33 and serine 46 in response to UV radiation (Bulavin, 1999).

This shows that different residues are phosphorylated by different proteins in response to different stresses, with all resulting in the stabilisation and activation of p53. Further modifications are discussed in detail in several reviews, such as (Appella and Anderson, 2001).

1.3.5. p53 and cancer

The role of p53 in cancer has been greatly studied. As one of the most mutated genes in cancer, it is unsurprising that p53 has been a key target in cancer research. Many studies have focused upon researching the role of mutant p53, and also reactivating p53 as a

cancer therapeutic option. It has been shown that loss of p53 function is often a requirement for maintaining tumours that are already established. A variety of studies showed that by reconstituting p53 in such tumours, tumour regression occurred (Roth *et al.*, 1996; Martins, Brown-Swigart and Evan, 2006; Ventura *et al.*, 2007; Feldser *et al.*, 2010; Junttila *et al.*, 2010; Xue *et al.*, 2011). In the clinic, gene therapy to reintroduce p53 through use of vectors, such as adenoviruses, has proved successful in cancer treatment (Senzer *et al.*, 2007). Additionally, small molecule drugs that activate and stabilise p53 have been developed with the view of enhancing treatment of cancer cells that retain wild type p53. In the 50% of tumours carrying wild type p53, often the function of p53 is lost due to increased activity of p53 inhibitors, such as MDM2. Therefore, pharmacological inhibition of MDM2 also seemed an attractive approach for tumours with wild type p53 (Vassilev, 2004). Nutlin-3, an MDM2 inhibitor, has been used in many studies to allow p53 activation in cancers (Kojima *et al.*, 2005; Sonnemann *et al.*, 2011; Knkele *et al.*, 2012). Other MDM2 inhibitors are also being tested for this purpose (Lakoma *et al.*, 2015; Lu *et al.*, 2016). A similar approach of reactivation of p53 is also being studied in the context of mutant p53. In these cases, the aim of restoration of p53, rather than being through inhibition of negative regulators, is to use small molecules to alter the folding of mutant p53 into a wild type conformation (Selivanova and Wiman, 2007).

Whilst these approaches show much promise, and enter clinical trials, there are also suggestions that in certain cases the presence of wild type p53 in tumours can actually have a negative outcome on the response to treatment. Some breast cancers retaining wild type p53 were shown to be protected from treatment with some cytotoxic drugs, and so in this case p53 was associated with a poor response to treatment (Bertheau *et al.*, 2008). Similarly, in certain cases p53 appears to cause a cell cycle arrest upon treatment with chemotherapeutic drugs, and results in protection of the cancer cells,

whilst p53 defective cells will undergo cell death. One such study showed that reducing the levels of the protein kinase ATM increased sensitivity to the poly (ADP-ribose) polymerase (PARP) inhibitor, olaparib, in gastric cancer cell lines that were p53 deficient, either through depletion or inactivation of p53 (Kubota *et al.*, 2014). Another study observed a similar effect in mantle cell lymphoma cells, with cells that were ATM and p53 deficient also showing increased sensitivity to olaparib (Williamson *et al.*, 2012). Further work from this group also showed that the ATM and p53 deficient sensitivity to olaparib also occurred in colorectal cancer cell lines (Wang *et al.*, 2017), showing that this effect was not cancer type specific. Another example of p53 deficient cancer cells showing increased sensitivity to chemotherapeutic drugs is in combination with checkpoint kinase 1 (Chk1) inhibitors. Use of A-690002 or A-641397, two Chk1 inhibitors, increased the cytotoxicity of both topoisomerase inhibitors and gamma-radiation in p53 deficient cells lines, but not p53 expressing cell lines, from different tissue origins (Chen *et al.*, 2006). Additionally, another study suggested that a p53 dependent arrest at G1 may protect cancer cells from undergoing cell death after treatment with ionising radiation and Chk1 inhibitors (Petersen *et al.*, 2010). Taking advantage of checkpoint defects in p53 deficient cancer cells may be a promising strategy for treatment of p53 deficient tumours.

As previously mentioned p53 has also been suggested to impact on the sensitivity of PLK1 inhibitors. Using siRNA depletion of PLK1 it was shown that cancer cells deficient of p53 showed increased mitotic arrest and aneuploidy and reduced cell survival compared to wild type p53 cancer cells (Guan *et al.*, 2005). This was supported by similar findings *in vitro* and *in vivo* that showed that cancer cells without wild type p53 were highly sensitive to PLK1 inhibitors (Sur *et al.*, 2009). Several other studies have observed similar effects, with increased sensitivity to PLK1 inhibition being

observed in p53 deficient cancers (Liu, Lei and Erikson, 2006; Degenhardt *et al.*, 2010; McKenzie *et al.*, 2010; Danovi *et al.*, 2013; Yim and Erikson, 2014).

As mutations in p53 are present in approximately 50% of cancers, and many cancer therapeutic options have been suggested to be more effective in p53 wild type tumours, the increased sensitivity of p53 deficient cancers to certain treatments, such as PLK1 inhibitors, could provide an opportunity to treat tumours that do not respond to other chemotherapeutics. This may allow an additional treatment plan that could potentially enhance the treatment of cancer patients.

1.4. Thesis Aims

As mentioned previously, upon treatment with PLK1 inhibitors there is an apparent p53 protective effect, with wild type p53 expressing cells showing a reduced sensitivity to PLK1 inhibitors in comparison to p53 deficient cells. Several studies have observed such findings. However, the mechanism by which p53 exerts such a protective effect is currently unknown. There is therefore an unmet need to better characterise this finding to allow improved efficacy of PLK1 inhibitors and enhanced treatment options. As a result, the aims of this thesis are:

- To determine whether previous findings of a p53 protective effect upon treatment with PLK1 inhibitors can be reproduced.
- Assuming confirmation of the protective effect, to investigate the underlying mechanism(s) and significance of the protective effect of p53 towards PLK1 inhibitors.

Chapter 2 : Materials and Methods

2.1. Reagents and Buffers

2.1.1. Antibiotics

Antibiotics	Manufacturer	Working Concentration	Use
Hygromycin B	Life Technologies	400 µg/ml	Selection of stable lines
Geneticin (G418)	Life Technologies	400 µg/ml	Selection of stable lines
Ampicillin	Melford	100 µg/ml	Bacterial selection
Kanamycin	Melford	50 µg/ml	Bacterial selection

Table 2.1. List of antibiotics used

2.1.2. Protein Expression Plasmids

DWM 902 – wild type human p53 open reading frame in pOPRSVICAT

DWM 903 – wild type human p53 open reading frame, containing serine 15 to alanine mutation, in pOPRSVICAT

DWM 947 – pCMVLacI repressor plasmid

2.1.3. Drugs Used in Cell Treatments

All drugs were stored at -20°C.

Drug	Manufacturer	Catalogue No.	Use
GSK461364	Selleckchem	S2193	PLK1 Inhibitor
BI6727	Selleckchem	S2235	PLK1 Inhibitor
S-Trityl-L-cysteine (STLC)	Sigma	164739	Eg5 Inhibitor
Etoposide	Selleckchem	S1225	Topoisomerase II Inhibitor
Paclitaxel (Taxol)	LC Labs	P-9600	Microtubule Stabilisation
Nocodazole	Millipore	487928	Prevents polymerisation of microtubules
IPTG	Melford	MB1008	Allolactose Molecular Mimic
KU-55933	Selleckchem	S1092	ATM Inhibitor
VE-821	Selleckchem	S8007	ATR Inhibitor
Z-VAD-FMK	Selleckchem	S7023	Pan-Caspase Inhibitor
5-Bromo-2'-deoxyuridine (BrdU)	Sigma	B9285	Thymidine Analogue
Nutlin-3	Tocris	3984	MDM2/p53 Interaction Inhibitor

Table 2.2. List of drugs used in mammalian cell treatments.

2.1.4. Primary Antibodies

Antibody	Manufacturer	Catalogue No.	Epitope	Dilution	Species
Actin	Sigma-Aldrich	A2066	Actin carboxy terminal (C11 peptide) (Polyclonal)	1:2000	Rabbit
BrdU Pure (B44)	Becton Dickinson	347580	Ioduridine (Monoclonal)	1:50	Mouse
BubR1	Bethyl Laboratories	A300-386A	Human BubR1 a.a. 350-400 (Polyclonal)	1:2000	Rabbit
BubR1 (Phospho-T680)	Abcam	ab200061	Human BubR1 a.a. 650-750 with phosphorylated T680 residue (Monoclonal)	1:2000	Rabbit
EG5/KIF11	Universal Biologicals	A301-076	Human Eg5 a.a. 1006-1056 (Polyclonal)	1:1000	Rabbit
Gamma H2A.X (Phospho-S139)	Abcam	ab11174	Human gamma H2A.X with phosphorylated serine 139 residue (Polyclonal)	1:5000	Rabbit
Gamma Tubulin	Sigma-Aldrich	T65557	a.a. 38-53 (Monoclonal)	1:1000	Mouse
GAPDH	Sigma-Aldrich	G8795	Rabbit GAPDH (Monoclonal)	1:5000	Mouse
Histone H3 (D1H2)	Cell Signaling Technology	4499	Human histone H3 carboxy terminus (Monoclonal)	1:2000	Rabbit
Histone H3	Millipore	06-570	Human histone H3	1:1000	Rabbit

(Phospho-S10)			with phosphorylated serine 10 residue (Polyclonal)		
KIF15	Universal Biologicals	A302-706A	Human Kif15 a.a. 1225-1275 (Polyclonal)	1:1000	Rabbit
MDM2 (4B2)	Moravian	4B2	Human MDM2 amino terminal (Monoclonal)	1:1000	Mouse
p21 (H-164)	Santa Cruz Biotechnology	sc-756	Human p21 a.a.1-164 (Polyclonal)	1:1000	Rabbit
p53 (DO-1)	Santa Cruz Biotechnology	sc-126	Human p53 amino terminal, a.a. 11-25 (Monoclonal)	1:1000	Mouse
p53 (Phospho-S15)	Cell Signaling Technology	9284	Human p53 with phosphorylated serine 15 residue (Polyclonal)	1:1000	Rabbit
PARP	Cell Signaling Technology	9542	PARP caspase cleavage site (Polyclonal)	1:1000	Rabbit
PLK1 (208G4)	Cell Signaling Technology	4513	Human PLK1 carboxy terminus (Monoclonal)	1:1000	Rabbit

Table 2.3. List of primary antibodies used for western blotting, flow cytometry and immunofluorescence.

2.1.5. Secondary Antibodies

Antibody	Manufacturer	Catalogue No.	Dilution	Species
Mouse IgG (whole molecule) – FITC	Sigma	F3008	1:64	Sheep
Rabbit IgG (whole molecule) – FITC	Sigma	F0382	1:80	Goat
Mouse IgG – HRP	Biorad	1721011	1:2000	Goat
Rabbit IgG – HRP	Biorad	1706515	1:2000	Goat
Mouse IgG (H+L) Alexa Fluor® 488	Life Technologies	A11029	1:1000	Goat

Table 2.4. List of secondary antibodies used for western blotting, flow cytometry and immunofluorescence.

2.1.6. Buffers and Solutions

Crystal Violet

0.4% (w/v) Crystal violet powder

20% (v/v) Ethanol

Enhanced Chemiluminescence (ECL) Solution 1

2.5 mM Luminol

100 mM Tris (pH 8.5)

396 μM P-Coumaric acid

Enhanced Chemiluminescence (ECL) Solution 2

0.0192% (v/v) H_2O_2

100 mM Tris (pH 8.5)

Flow Cytometry Antibody Buffer

1X PBS

0.5% (w/v) Bovine serum albumin

0.5% (v/v) Tween 20

Immunofluorescence Blocking Solution

1X TBS

5% (w/v) Bovine serum albumin

0.1% (v/v) Triton X-100

Phosphate Buffered Saline (PBS) (pH 7.4)

137 mM Sodium chloride

10 mM Phosphate

2.7 mM Potassium chloride

Ponceau S Staining Buffer

0.2% (w/v) Ponceau S

5% (v/v) Glacial acetic acid

Propidium Iodide Staining Buffer

50 µg/ml Propidium iodide

200 µg/ml RNase A

1X PBS

Protein Fixative

10% (v/v) Methanol

10% (v/v) Acetic acid

SDS Protein Sample Buffer (2X)

0.125 M Tris-HCl (pH 6.8)

20% (v/v) Glycerol

4% (w/v) SDS

0.02% (w/v) Bromophenol blue

SDS-PAGE Running Buffer (10X)

250 mM Tris

1.92 M Glycine

1% (w/v) SDS

TAE Buffer (50X)

2 M Tris

5.7% (v/v) Glacial acetic acid

50 mM EDTA (pH 8.0)

Western Blot Blocking Buffer

1X PBS

0.1% (v/v) Tween 20

5% (w/v) Marvel dried milk

Western Blot Transfer Buffer

25 mM Tris

192 mM Glycine

20% (v/v) Methanol

Western Blot Washing Buffer

1X PBS

0.1% (v/v) Tween 20

2.2. Cell Culture

2.2.1. Maintenance of Cells

All cell lines were cultured at 37°C in a humidified 5% CO_2 incubator. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS) (Biosera (Labtech International Ltd.)) and 2 mM L-glutamine (Gibco) was used to culture all cancer cell lines. When cells reached 70-80% confluency they were passaged. Cells were washed twice in PBS, before being detached by 1 ml 0.5% (w/v) Trypsin (Gibco), a dissociation agent. Cells were then resuspended in 9 ml medium and transferred to new flasks (1:10) with additional fresh medium.

Cell Line	ATCC® Number	Tissue and Disease	p53 Status	Morphology
HCT116 p53+/+	CCL-247	Human colorectal carcinoma	p53 wildtype	Epithelial
HCT116 p53-/-	Vogelstein Laboratory	Human colorectal carcinoma	p53 null	Epithelial
MCF-7	HTB-22	Human mammary gland, breast adenocarcinoma	p53 wildtype	Epithelial
MCF-7 p53 KO	Buluwela Laboratory	Human mammary gland, breast adenocarcinoma	p53 null	Epithelial
U2OS	HTB-96	Human osteosarcoma	p53 wildtype	Epithelial
H1299	CRL-5803	Human non-small cell lung carcinoma	p53 null	Epithelial

Table 2.5. Mammalian cell lines used.

2.2.2. Storage of Cell Lines

All cell lines were stored in freezing medium (FBS + 10% (v/v) DMSO) in liquid nitrogen. Cells were trypsinised and resuspended in medium before being centrifuged at 300 RCF for three minutes. The supernatant was then aspirated and the cell pellet was resuspended in freezing medium. 1 ml aliquots of the cell suspension were pipetted into labelled cryotubes. These were then transported to the -80°C freezer on ice, where they were slowly frozen in specialised freezing boxes. After a few days they were transported on dry ice to liquid nitrogen.

To bring the cells back into culture, they were quickly thawed at 37°C in a water bath. They were then centrifuged gently, the freezing medium was removed and they were resuspended in DMEM media before addition to a fresh flask.

2.2.3. Plating Cells for Experiments

Cells to be seeded for experiments were obtained during passaging of cells as described above. The resuspended cells were collected and 10 µl of the total 10 ml suspension was added to a haemocytometer (Hawksley, 1/400 m^2). Cells were counted under the microscope and the stock solution of cells was diluted and plated as required for each experiment.

2.2.4. MTS Assay

To assess the dose response of different drugs on different cell lines an MTS cell viability assay was used. 2500 cells/well were seeded in 96-well plates in triplicate for each condition. The following day cells were left untreated, treated with DMSO (vehicle control) or treated with 2.5, 5, 10, 20 or 40 nM concentration of GSK461364, BI6727 or

Taxol or 0.625, 0.125, 0.25, 0.5, 1 or 2 μM STLC, in a total volume of 100 μl per well. After 68 hours of exposure to the drug, 20 μl of CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) reagent was added and the absorbance was read 4 hours later (72 hours total treatment) at 560 nM using the GloMax®-Multi Detection System (Promega).

2.2.5. Colony Formation Assay

In order to determine the ability of cells to recover post treatment a colony formation assay was used. $5x10^4$ cells were seeded in 6-well plates in duplicate. The following day cells were treated with a range of concentrations of GSK461364, BI6727 or Taxol for 72 hours. After treatment, the media was removed and replaced with fresh media. Cells were then allowed to grow for a further 10 days, with media being replenished every 2-3 days. After 10 days the cells were washed twice with 1X PBS, fixed with protein fixative, stained with 0.4% crystal violet solution and finally washed 4 times with dH_2O. Resulting colonies could then be visualised.

2.2.6. Flow Cytometry (S phase labelling)

To allow analysis of differences to the cell cycle distribution upon treatment with drugs, flow cytometry was used. 5-bromo-2-deoxyuridine and propidium iodide were used to stain the cells to allow determination of the percentage of cells in each stage of the cell cycle at the time of harvesting. Cells were seeded in 6 cm plates and treated with the desired drug(s). At the treatment end point, cells were labelled with 30 μM BrdU, added directly to the media and incubated for 30 minutes. Media was collected, followed by washing the cells twice with PBS with each wash being collected also. The cells were then trypsinised and pooled together with the supernatant. Cells were then pelleted for five minutes at 300 RCF, washed once with PBS, pelleted again then resuspended in 1

ml PBS. This suspension was then added to 3 ml of ice-cold ethanol while vortexing to prevent clumping of the cells. The cells were then kept at 4°C for at least 30 minutes and up to two weeks.

Fixed cells were then subsequently stained. Two washes in PBS-1% FBS were carried out, before treatment with 2 M HCl for 20 minutes in a 37°C water bath. During this time the cells were gently but effectively shaken. Two washes in PBS-1% FBS then followed. The pelleted cells were resuspended in 200 µl of anti-BrdU antibody diluted 1 in 50 in flow cytometry antibody buffer and incubated for one hour at room temperature in the dark. One wash in PBS-1% FBS followed, before resuspension of the cell pellet in 200 µl Anti-Mouse IgG (whole molecule)–FITC (Fluorescein isothiocyanate) antibody produced in sheep diluted 1 in 64 in flow cytometry antibody buffer. The cells were then incubated at room temperature in the dark for 30 minutes. Finally, one more wash in PBS-1% FBS was carried out before resuspending the cell pellet in 300 µl of propidium iodide staining buffer in FACS tubes (Becton Dickinson). Cells were then analysed on the Becton Dickinson FACS Canto Flow Cytometer. Analysis of the data was carried out using Flowjo software.

2.2.7. Flow Cytometry (Mitotic cell labelling)

Flow cytometry with propidium iodide alone does not allow discrimination between cells in G2 and cells in mitosis. However, use of an antibody detecting a protein that is specific to mitosis allows separation of mitotic cells from G2 cells. Histone H3 phosphorylation at serine 10 is a well established modification that occurs at the onset of mitosis, and so use of an antibody to detect this phosphorylation, along with a fluorescently tagged secondary antibody, allows quantification of the mitotic index of a population of cells through use of flow cytometry. To do this, cells were seeded at the

appropriate density in 6 cm dishes, and treated the following day. At the treatment end point (4 or 8 hours), the media was collected from the cells and added to a falcon tube. The cells were washed twice with PBS, with the washes also being retained, before cells were harvested through trypsinisation. Cells were then added to the falcon tube, so that both adherent and floating cells could be analysed. After centrifugation at 300 RCF, cells were washed once in PBS before fixation in 0.5% (w/v) paraformaldehyde for 20 minutes at 37°C. Cells were washed once in PBS before resuspension in ice cold 70% ethanol and storage at -20°C. Cells were stored for one to three days before staining was carried out.

To stain cells, the cells were allowed to warm to room temperature, washed twice with PBS–1% FBS, and the resulting pellet was resuspended in 100 µl of flow cytometry antibody buffer containing phosphorylated histone H3 (serine 10) at a dilution of 1:100. The cells were incubated at room temperature for one hour, before washing in PBS–1% FBS, and then a further 30 minutes incubation at room temperature in the dark in flow cytometry antibody buffer containing rabbit IgG (whole molecule)–FITC antibody at a dilution of 1:80. Cells were washed once again in PBS–1% FBS, before resuspension in 300 µl of propidium iodide staining buffer in FACS tubes (Becton Dickinson). Cells were then analysed on the Becton Dickinson FACS Canto Flow Cytometer and Flowjo software was used to analyse the resulting data.

2.2.8. siRNA Transfection

PLK1 and p53 siRNA transfections were performed using a reverse transfection procedure. As a control, a non-silencing siRNA was used as a comparison. The siRNAs used are described in table 2.6. 1.5 µl of 20 µM siRNA was added to 500 µl Opti-MEM I Reduced Serum Media (Gibco) in a 6-well plate. 5 µl of Lipofectamine® RNAiMAX

(Invitrogen) was then added, mixed and incubated at room temperature for 20 minutes. Cells were trypsinised, counted and diluted to $1x10^5$. Following the 20 minutes incubation time 2.5 ml of cells were added to the well (total cell density of $2.5x10^5$ cells per well). Cells were incubated for 24 - 48 hours before harvesting/ further treatments were carried out.

Target	Sequence	Manufacturer	Catalogue No.
p53 (exon 7)	5'-GACUCCAGUGGUAAUCUACUU-3'	Thermo Scientific	OSLR-001137
PLK1	5'-GCACAUACCGCCUGAGUCU-3' 5'-CCACCAAGGUUUUCGAUUG-3' 5'-GCUCUUCAAUGACUCAACA-3' 5'-UCUCAAGGCCUCCUAAUAG-3'	Thermo Scientific	L-003290-00
Non-silencing	5'-CAGUCGCGUUUGCGACUGGUU-3'	Thermo Scientific	OSLR-001139

Table 2.6. siRNA used in cell culture.

2.2.9. Synchronisation by Serum Starvation

Cells were seeded in complete growth media in 6 cm plates and left overnight to adhere. The following day, cells were washed three times in PBS before addition of DMEM media containing 0.1% (v/v) FBS. Cells were incubated for 24 hours in the reduced serum media to synchronise cells in G0. After 24 hours complete growth media was added to stimulate synchronous entry of cells back into cycle. Cells were subsequently harvested/treated at different time points as described.

2.2.10. Live Cell Imaging (Phase)

Cells were seeded in 24-well plates to achieve 80% confluency at the time of treatment. Desired treatments were added, e.g. GSK461364 or BI6727 and imaging then took

place in a heated chamber (37 °C and 5% CO2) using a 10X/03 objective lens on a Zeiss Axiovert 200M microscope controlled by Micro-Manager software. Positions were selected in each well and after approximately one hour the focus was set. This allowed the temperature and CO_2 to be maintained, thus ensuring the cells were settled to help prevent the focus drifting. Images were taken using a C4742-80-12AG camera (Hamamatsu) and were captured every four minutes, with 250 images being recorded for each position (approximately 16 hours total time). Image J (National Institutes of Health) software was then used to manually calculate the time in mitosis, with the start time being the rounding of the cell and the end time being the decision of cellular fate. For each condition 50 cells were analysed.

2.2.11. Live Cell Imaging (Fluorescence)

Cells were seeded in μ-slide 8-well chambers (Ibidi) and left overnight to adhere. The following day, DMEM media was removed and replaced with carbon dioxide independent Leibovitz's L-15 media (Gibco) supplemented with 10% (v/v) FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin (Gibco). After approximately four hours the L-15 media was removed and 100 nM SiR-DNA (tebu-bio), diluted in L-15, was added to the cells for 15 minutes. Cells were then washed once with PBS before addition of L-15 media containing the drugs of interest. The plate was then mounted on the incubated chamber of the Deltavision Elite microscope controlled by SoftWoRx software. Cells were imaged using a 40X/1.30NA U Plan FLN oil objective lens with images being captured on the CoolSNAP HQ2 camera (Photometrics). The Cy5 channel (imaging of far red fluorescence) was used to image the SiR-DNA and a reference image in phase contrast allowed the cells to be imaged. Images were taken every 4 minutes for approximately 250 frames, with 4 optical sections being captured at 5 μm optical spacings. Image J software was again used to analyse the duration of mitosis. In

this instance the DNA was analysed. The entry to mitosis was determined as the point at which the DNA condensed, and the exit from mitosis was the point at which the DNA decondensed after chromosome segregation. These times were plotted as box plots to allow quantitative differences to be easily observed.

2.2.12. Antibiotic Dose Selection for Stable Lines

In order to determine the concentration of antibiotic required for selection of transfected cells, cells were seeded in 24-well plates. Increasing doses of antibiotic (G418 or Hygromycin B) were added to the cells in DMEM media, with the range normally being 0, 200, 400, 600, 800 and 1000 μg/ml. The antibiotic and media were replaced every 2-3 days. After 1 to 2 weeks of treatment, the lowest concentration of antibiotic that resulted in complete cell death was chosen as the required dose for selection.

2.2.13. Production of H1299 p53 S15 and S15A IPTG Inducible Cell Lines

H1299 cells were seeded at a density of $1x10^6$ per 10 cm plate. 1 hour prior to transfection cells were washed with 5 ml Opti-MEM, and incubated at 37°C in a further 5 ml of Opti-MEM. For each plate, 400 μl of Opti-MEM was mixed with the required volume of plasmid DNA. 100 ng of each plasmid was used, with DWM 902 or DWM 903 both being added with DWM 947. A second 400 μl of Opti-MEM was mixed with 20 μl Lipofectamine® 2000. After five minutes of incubation at room temperature, the two solutions were combined and mixed. A further 20 minutes incubation followed before adding the transfection mix drop wise to the cells. After approximately six hours a further 5 ml of Opti-MEM was added to each plate. The cells were incubated overnight, then the following day the media was removed and 10 ml fresh DMEM was added to each plate.

After a further 24 hours the cells were put into antibiotic selection. Geneticin (G418) and Hygromycin B were used at 400 ug/ml concentrations. The selection was maintained for two weeks, with renewal of media/ antibiotics every 2-3 days. After two weeks colonies were big enough to be seen by eye. The media was removed and the plate was washed twice with PBS. A p20 Gilson pipette was then used to pick individual colonies. Approximately 10 µl of trypsin was added to each colony and by use of scraping and pipetting the colony could be lifted from the plate. The colony was then transferred to a 24-well plate with antibiotic selection being maintained. Colonies were continually expanded and cells were frozen down for storage and also harvested for screening to ensure they were positive for both plasmids. Once positive colonies were obtained, the cells could be used for further experiments.

2.2.14. Induction of p53 in H1299 S15 and S15A Inducible Cell Lines

Cells were seeded in 6-well plates or 6 cm dishes. The following day IPTG was added to the cells in fresh DMEM media. For testing induction different concentrations of IPTG were used, thereafter 100 µM IPTG was used. The IPTG was left for 16 hours before harvesting cells or further treatments were carried out, with IPTG being maintained throughout the experiments.

2.2.15. Immunofluorescence (Centrosome Staining)

PLK1 is involved in the maturation and separation of centrosomes, so to investigate whether the presence of p53 has an effect on the movement of centrosomes immunofluorescence with a gamma-tubulin antibody was used. Cells were seeded on poly-L-lysine coated coverslips in 24-well plates. The following day treatments were carried out. At the treatment end point cells were washed twice in PBS then fixed on the

coverslips using 4% paraformaldehyde solution for 15 minutes. Two washes in PBS were then carried out, before addition of 0.1% (v/v) Triton X-100 in PBS for five minutes. A further three washes in PBS were completed, then immunofluorescence blocking solution was added for 15 minutes at room temperature. Primary antibody (γ-tubulin) was then diluted in immunofluorescence blocking solution. As a control, blocking solution without any antibody was also used. The antibody was then added to the coverslips and incubated at room temperature for one hour. Coverslips were then washed three times for five minutes each with PBS. Secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse IgG) was then diluted in immunofluorescence blocking solution for one hour at room temperature. A further three five minute washes were carried out with PBS before staining with DAPI. DAPI was diluted 1:10000 in immunofluorescence blocking solution and added to the coverslips for five minutes at room temperature. Finally, four washes with PBS were completed and then the coverslips were mounted onto slides using ProLong® Gold Antifade Mountant (Life Technologies). The samples were left to solidify for 24 hours at room temperature and then stored at 4°C. The Leica AF6000 wide field fluorescent microscope controlled by LAS AF software was used to acquire images using a 63x oil objective and Leica DFC420 camera.

2.2.16. Determination of Resistance in Surviving Cells

To address the possibility that the proportion of cells surviving treatment with PLK1 inhibitors are a resistant population, double treatments were used. Cells were treated for 72 hours with 5, 7.5, 10, 15 or 20 nM of GSK461364 or BI672. The drug was then removed and cells were allowed to recover. Once enough cells were obtained (approximately a 70-80% confluent 6 cm plate), the surviving cells were plated for further experiments. To determine if the cells were resistant to PLK1 inhibitors MTS

and colony formation survival assays were carried out. 2500 cells were seeded per well in 96-well plates for the MTS viability assay. Each respective surviving concentration was plated along with cells that had never been exposed to PLK1 inhibitors as a control. The day after seeding, cells were treated with increasing doses of the respective PLK1 inhibitor (0, 2.5, 5, 10, 20, 40 or 80 nM), and incubated for 68 hours before addition of MTS reagent for a further four hours. The absorbance was then determined at 560 nm using the GloMax®-Multi Detection System (Promega). For the colony formation assay cells were seeded in 6-well plates, treated with a range of concentrations of PLK1 inhibitors and incubated for 72 hours. The drug was then removed and cells were allowed to recover in fresh media. After 10-12 days the cells were stained with crystal violet and resulting colonies were visualised.

2.3. Protein Biology Techniques

2.3.1. Preparation of Samples for SDS-PAGE and Western Blotting

In order to determine differences in protein levels western blotting was used. Cells were seeded in 6-well or 6 cm plates and treatments were carried out as indicated in the figure legends. At the treatment end point the cells were placed on ice. The media was collected in order to retain floating cells, which could be apoptotic or mitotic cells, and this was centrifuged at 300 RCF for five minutes at 4°C. 1 ml of 1X PBS was added to the well/plate containing the adherent cells. A cell scraper was used to remove cells from the plate and put them into suspension. This suspension was transferred to a microcentrifuge tube and kept on ice. The supernatant from the pellet of floating cells was aspirated then the pellet was resuspended in 100 µl of PBS and transferred to the microcentrifuge tube containing the equivalent sample of adherent cells. This was centrifuged at 10000 RCF for five minutes at 4°C, before washing in 1X PBS. The

supernatant was then aspirated and the pellet was lysed. 250-400 µl (depending on pellet size) of 2X SDS protein sample buffer was added to lyse the pellet, with a 15 minute incubation period on ice following. The sample was then sonicated for 20 seconds at 30% amplitude, before centrifugation at 10000 RCF for five minutes. The Bio-Rad DC Protein Assay was used to quantify the protein concentration with absorbance being measured using the GloMax®-Multi Detection System (Promega). The sample was then diluted in 2X SDS protein sample buffer to give a concentration of 1 µg/µl and DTT was added to a final concentration of 0.1 M. Samples were then boiled for five minutes at 100°C, centrifuged and were then ready to load onto gels as described below.

2.3.2. SDS-PAGE

To separate proteins SDS-PAGE was used with either 8% or 10% polyacrylamide gels, dependent on the molecular weight of the protein of interest. Both 8% and 10% resolving gels comprised 382 mM Tris (pH 8.8), 0.1% (v/v) SDS, 0.1% (v/v) APS and 1 µl/ml N,N,N,N'-Tetramethylethylenediamine (T.E.M.E.D.). Only the acrylamide concentration differed between the 8% and 10% gels with acrylamide being added at the desired concentration from a 30% (w/v) acrylamide stock (Severn Biotech Ltd.). The resolving gel was poured first, leaving enough space for the well comb to be inserted later. This gap was filled with 70% (v/v) ethanol, to remove any bubbles whilst the gel set. After waiting at least 10 minutes to allow the gel to set, the ethanol was poured off, and the stacking gel was added. The stacking gel was composed of 4.9% (v/v) acrylamide, 123 mM Tris (pH 6.8), 0.1% (v/v) SDS, 0.1% (v/v) APS and 1.63 µl/ml T.E.M.E.D. As soon as the stacking gel was poured, a well comb was added to create the wells required for addition of samples. Once set, the gels were inserted into a vertical slab electrophoresis chamber (Atto) with 1X SDS-PAGE Running Buffer. 20 µg of protein sample was loaded onto the gel, along with 4 µg of PageRuler™ Prestained

Protein Ladder (ThermoFisher Scientific). The gel was then run at 100 V until the dye front reached the bottom of the gel.

2.3.3. Western Blotting

To transfer the proteins separated by SDS-PAGE, western blotting was used. The gel was put into a 'sandwich' with Whatman filter paper (Fisher Scientific), sponges and Hybond ECL nitrocellulose membrane (GE Healthcare). Transfer was then carried out in a Mini Trans-Blot® Cell (Bio-Rad) with western blot transfer buffer for one hour at 100 V. The membrane was then stained with Ponceau S staining buffer to detect the presence of proteins on the membrane. After rinsing with water, the membrane was blocked on a roller for one hour at room temperature in western blot blocking buffer. Primary antibody was then added to Western blot blocking buffer at the dilution indicated in table 2.3 before addition to the membrane and overnight incubation at 4°C on a roller.

The following morning the membrane was washed three times at five minutes each in PBST then the appropriate secondary antibody (either mouse or rabbit horseradish peroxidase-conjugated secondary antibody) was added to the membrane in blocking buffer and incubated for one hour at room temperature. A further three five minute washes were carried out before addition of enhanced chemilluminescence reagent 1 and reagent 2, mixed in a 1:1 ratio, to allow complete and even coverage of the membrane. The ChemiDoc™ MP Imaging System (Bio-Rad) controlled by Image Lab 4.1 software was then used to visualise the membrane.

2.4. Molecular Biology Techniques

2.4.1. Transformation of Bacteria

Competent *E.coli* bacteria (DH5α) were used for transformation of plasmid DNA into bacterial cells. A frozen aliquot of DH5α was allowed to thaw on ice. 100 µl of DH5α competent cells were added to 0.5 µg of plasmid DNA in a microcentrifuge tube. Gentle tapping was used to mix the solution of DNA and bacteria, before incubation on ice for approximately 12 minutes. The bacteria were then heat shocked at 37°C in a water bath for five minutes. The tube was then put on ice again, before addition of 1 ml of LB containing the appropriate antibiotic. The mixture was then incubated at 37°C for one hour on a shaker. LB agar plates containing the appropriate antibiotic (ampicillin or kanamycin) were prepared. After one hour of incubation, 100 µl of the transformation mixture was spread evenly over the agar plate. The plate was then incubated overnight at 37°C in an inverted position and the following morning the plate was checked for colonies. A colony could then be picked and added to LB for overnight growth at 37°C, or the plate could be stored at 4°C.

2.4.2. Glycerol Stocks

Plasmids were stored as bacterial glycerol stocks at -80°C. After transformation of plasmid DNA in competent bacterial cells (described above) the transformed bacteria were grown overnight in 4 ml LB, including the appropriate antibiotic, at 37°C with shaking. The following morning, 800 µl of the suspension was added to 300 µl 100% glycerol in a cryotube. The mixture was then snap frozen on dry ice before long term storage at -80°C.

2.4.3. Obtaining Plasmid DNA from Glycerol Stocks

Depending on the amount of plasmid required, a small or large culture could be grown. In the case of a small culture, 4 µl of antibiotic was added to 4 ml LB, and for a large culture 400 µl of antibiotic was added to 400 ml LB. A stab was taken from the glycerol stock and added to the LB. The bacteria were then grown overnight at 37°C with agitation. If a small culture was used, 1.5 ml of the solution was added to a microcentrifuge tube. The solution was then centrifuged, and the plasmid DNA was recovered using the QIAprep Spin Miniprep Kit (Qiagen), carried out as described by the manufacturer's instructions. If a large culture was used, all of the bacterial solution was pelleted. The supernatant was then poured off and the resulting pellet was used for recovery of the plasmid DNA by following the manufacturer's instructions on the QIAprep Spin Maxiprep Kit (Qiagen). Following completion of the maxi or mini prep kit, the concentration of the resulting plasmid DNA could be measured using a NanoDrop.

2.4.4. Agarose gel electrophoresis

In order to separate DNA obtained from maxi or mini preps, agarose gel electrophoresis was used. A 1% agarose gel was made with 1X TAE buffer, and 0.5 µg/ml ethidium bromide. The ethidium bromide allowed visualisation of DNA, through its intercalation to DNA and its fluorescent properties. Once the gel had set, it was assembled in a horizontal running chamber (Atto), and submerged in 1X TAE buffer. DNA loading buffer was then added to the DNA samples and loaded to the gel, along with DNA ladder, before running at 100V. Once the gel had successfully run, the gel was removed from the chamber and the ChemiDoc™ MP Imaging System (Bio-Rad) controlled by Image Lab 4.1 software was used to visualise the gel.

Chapter 3 : p53 reduces sensitivity to PLK1 inhibitors

3.1. Background

Previous work in the Meek lab has shown that p53 has a substantial effect on the levels of PLK1 (McKenzie *et al.*, 2010) and some reports suggest that in the absence of p53, levels of PLK1 increase (Sur *et al.*, 2009). Using PLK1 knockdowns Guan et al. (2005) showed that mitotic arrest and apoptosis was enhanced in cancer cells with defective p53. Further reports have also suggested that p53 can have a negative influence on the outcome of treatment with PLK1 inhibitors (Liu, Lei and Erikson, 2006; Sur *et al.*, 2009; Degenhardt *et al.*, 2010; McKenzie *et al.*, 2010; Danovi *et al.*, 2013; Yim and Erikson, 2014). As p53 is lost or mutated in around 50% of cancers, and thus 50% of cancers retain wild type p53, it is of great importance to know if p53 has an effect. If use of PLK1 inhibitors will only be beneficial to cancers that have either lost p53 or have a mutation in p53, then the p53 status of the cancer should first be assessed before treatment commences. Therefore, this chapter aims to confirm whether p53 plays a role in influencing the resistance to PLK1 inhibition as a cancer therapeutic treatment.

In this chapter, established cells lines were used to investigate the impact of p53 upon treatment with PLK1 inhibitors. This included the HCT116 cell line, which is a colon cancer-derived cell line that expresses wild type p53. However, an isogenic line in which full-length p53 has been removed through targeted homologous recombination was also used (Bunz *et al.*, 1998). This provides a highly useful tool for assessing the roles of p53, as the only difference between these lines is the p53 status.

3.2. Aim

The aim of this chapter is to determine whether p53 offers a protective effect upon treatment with PLK1 inhibitors.

3.3. Results

3.3.1 p53 increases resistance to treatment with PLK1 inhibitors

As previously discussed, there has been much debate about the involvement of p53 in the outcome of treatment with PLK1 inhibitors. The first starting point was therefore to test whether p53 has a role on the efficacy of PLK1 inhibitors. In order to address the question of whether p53 offers a protective effect upon treatment with PLK1 inhibitors, two independent PLK1 inhibitors developed by GlaxoSmithKline and Boehringer Ingelheim, GSK461364 and BI6727 respectively, were used. Use of two independently developed inhibitors targeting the same protein reduces the risk that observed results are due to off target effects. Two approaches of determining cell survival were used: a short-term MTS assay and a longer-term colony formation assay. The MTS assay, a colorimetric assay based on the reduction of MTS tetrazolium by viable cells to form a coloured formazan, with the resulting absorbance being read, was used with different doses of PLK1 inhibitors (0-40 nM). The HCT116 parental line (p53+/+), and a derivative line lacking full-length p53 expression (p53-/-) were seeded in 96-well plates in triplicate for each condition tested. As can be seen in Figure 3.1 both drugs reduced cell viability in a dose dependent fashion. However, the wild type p53 cells have increased resistance to both PLK1 inhibitors compared to the p53-/- cells, suggesting wild type p53 is contributing to the efficacy of these inhibitors. This is consistent with previous studies that suggest that p53 provides a protective effect upon inhibition of PLK1 (Guan *et al.*, 2005; Liu, Lei and Erikson, 2006; Sur *et al.*, 2009; Degenhardt *et al.*, 2010; McKenzie *et al.*, 2010; Danovi *et al.*, 2013; Yim and Erikson, 2014).

The colony formation recovery assay also used different doses of PLK1 inhibitors, with 72 hours of treatment, followed by removal of the drug and an additional 10 days of recovery in fresh media. The resulting colonies were fixed and then stained with crystal

violet to allow visual representation of the survival. As can be seen in Figure 3.2 the cells were killed in a dose dependent manner, reflecting the results of the MTS assay. Again, when treated for 72 hours the p53+/+ cells showed increased recovery compared to the p53-/- cells, supporting the results of the MTS viability assay.

One possibility of the p53 protective effect was that any form of inhibition of mitosis, not specifically PLK1, would result in this outcome. In order to address this Taxol, a microtubule poison that prevents the depolymerisation of microtubules resulting in a mitotic arrest at the metaphase/anaphase boundary, was used in both the MTS and colony formation assays with the HCT116 lines. Figure 3.3 shows that in the case of Taxol, absence of p53 does not increase sensitivity in the MTS assay. In the colony formation assay there is a slight increase in the number of surviving cells in the HCT116 p53-/- cell line compared to the p53+/+ cell line. This is not the same as observed with PLK1 inhibitors. Overall, this suggested that the p53 protective effect was a consequence of a PLK1 targeted mitotic arrest rather than a general mitotic arrest.

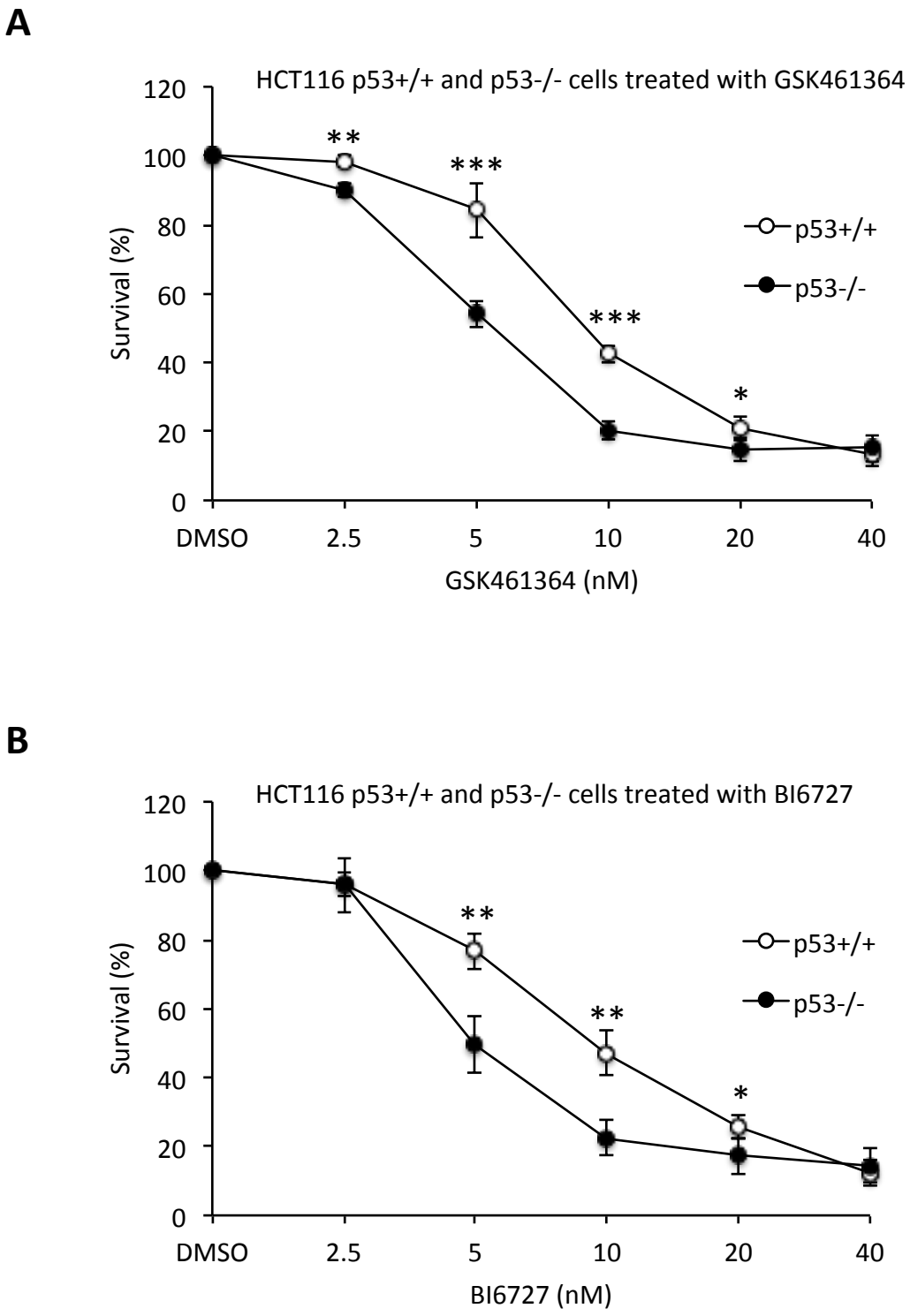


Figure 3.1. Cells expressing wild type p53 show reduced sensitivity to the independently developed PLK1 inhibitors, GSK461364 and BI6727. HCT116 p53+/+ and p53-/- cells were treated for 72 hours with increasing concentrations of the PLK1 inhibitor compounds, GSK461364 (A) or BI6727 (B). Cell viability was then measured using an MTS assay. A Student's t-test was used to evaluate significance of survival between p53+/+ and p53-/- cells at each concentration of PLK1 inhibitor. Experiments were performed with five biological replicates and three technical replicates. *p<0.05; **p<0.01;***p<0.001.

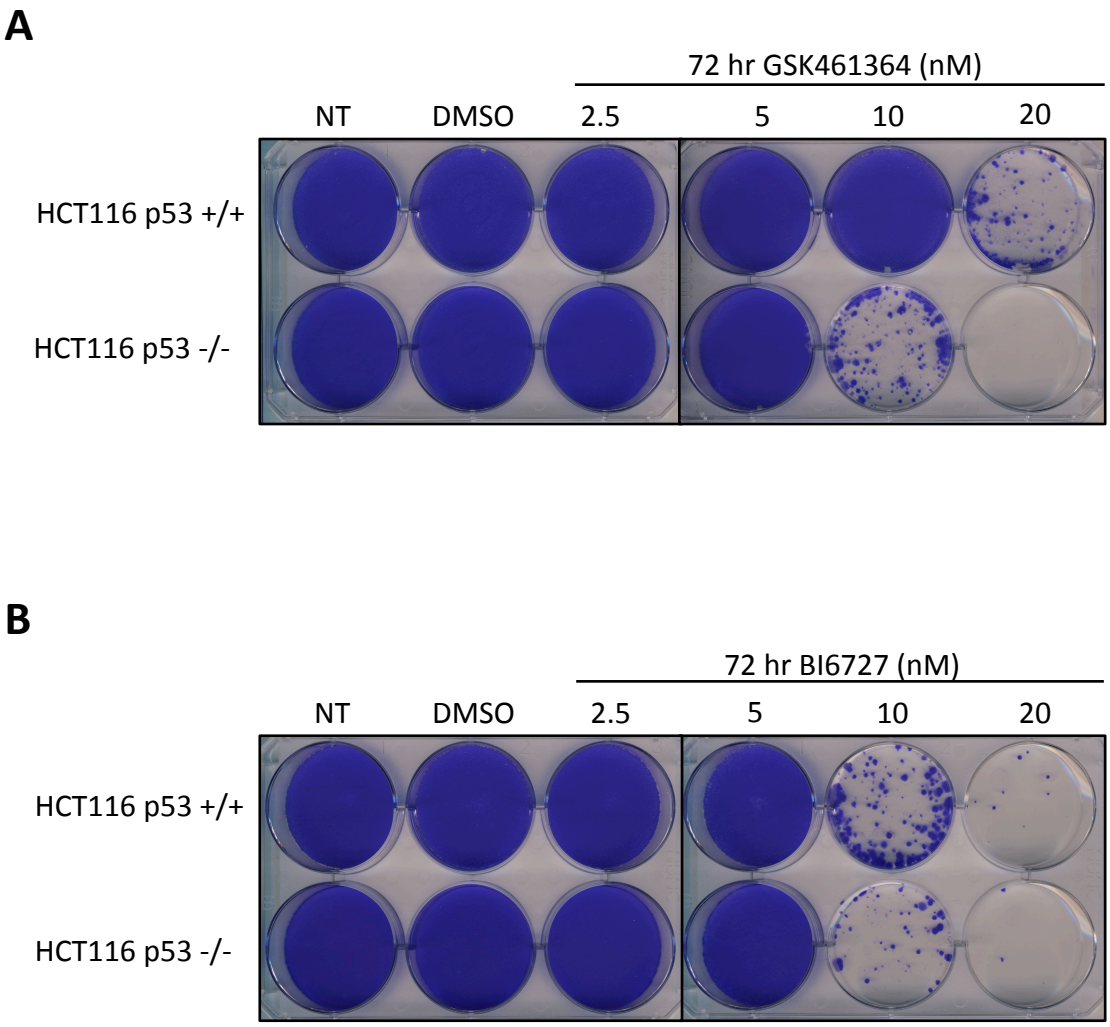


Figure 3.2. HCT116 p53+/+ and p53-/- cells treated with PLK1 inhibitors. HCT116 p53+/+ and p53-/- cells were treated with increasing doses of PLK1 inhibitors GSK461364 (A) or BI6727 (B) for 72 hours. The drug was then removed and the cells were allowed to recover in fresh media for a further 10 days. Cells were then washed in PBS, fixed in protein fixative and stained with crystal violet. The data are representative of three independent experiments each conducted in duplicate.

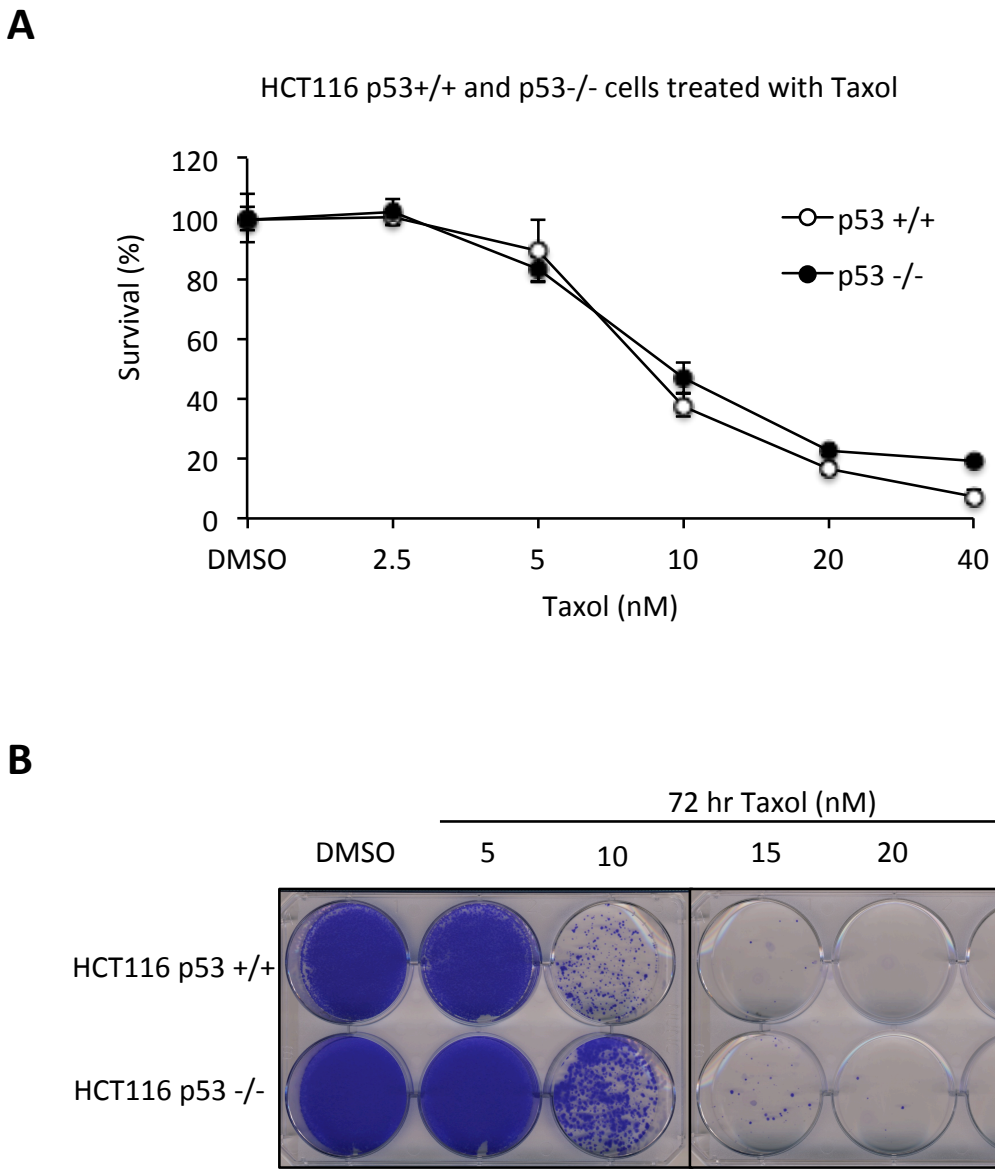


Figure 3.3. The outcomes of treatment of cells with Taxol is independent of the presence of p53. (A) HCT116 p53+/+ and p53-/- cells were treated for 72 h with DMSO, 2.5, 5, 10, 20 or 40 nM Taxol. Cell viability was then measured using an MTS assay. The data are representative of three independent experiments each conducted in triplicate. (B) HCT116 p53+/+ or p53-/- cells were treated for 72 h with increasing concentrations of Taxol. Following removal of the drug, cells were grown for a further 10 days. Surviving cells/colonies were detected following removal of the medium, fixing of the cells and staining with crystal violet. The data are representative of three independent experiments.

3.3.2 Cells surviving treatment with PLK1 inhibitors are not a resistant population

The appearance of cells that survive treatment with GSK461364 and BI6727 could indicate that there is a resistant population of cells within the HCT116 line, giving rise to the surviving colonies. In order to address this possibility the surviving cells were tested for resistance. To do this HCT116 p53+/+ and p53-/- cells were treated with 5, 7.5, 10, 15 or 20 nM of GSK461364 for 72 hours. The drug was then washed off and the cells were allowed to recover in fresh media. Once the surviving cells were around 70 - 80% confluent, they were pooled and expanded into a 6 cm dish to allow enough cells to set up further experiments. When enough cells were obtained they were collected and counted. These cells were then plated for MTS and colony formation assays. As a control, parental HCT116 p53+/+ and p53-/- cells that had not been exposed to any PLK1 inhibitor were also plated. It was expected that if resistant populations existed then cells surviving and recovering from treatment with a particular concentration of PLK1 inhibitor would then survive the second round of treatment at the same dose.

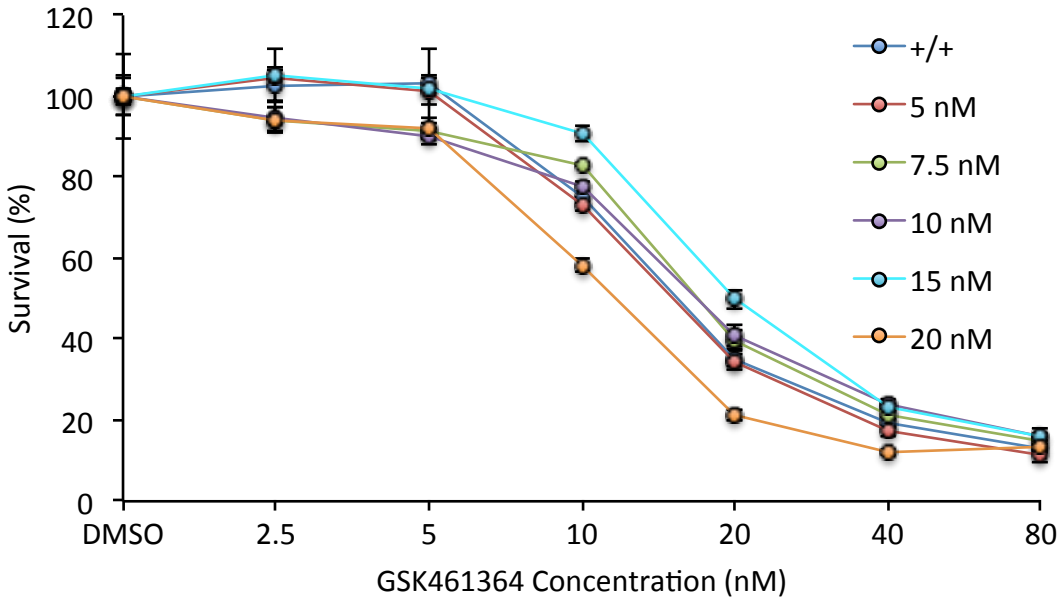
For the MTS assay, 2500 cells were seeded per well in 96-well plates. The HCT116 p53+/+ and p53-/- parental cells, and cells surviving 5, 7.5, 10, 15 and 20 nM treatments of GSK461364 were seeded in triplicate. These cells were then treated with DMSO or treated with 2.5, 5, 10, 20, 40 or 80 nM of GSK461364. The viability of cells after exposure to the drug for 72 hours was then measured. The resulting graphs are shown in Figure 3.4 A and C. As can be seen, there was very little difference between the different populations of cells, with no increase in resistance of cells that had previously been treated. This suggested that there is not a resistant population of cells within the HCT116 p53+/+ or p53-/- line.

Next the colony formation assay was used. Again the same populations of cells were seeded in 6-well plates, and then treated with DMSO, 5, 7.5, 10, 15 or 20 nM of GSK461364. The cells were exposed to the drug for 72 hours before being allowed to recover in fresh media for a further 10 days. Figure 3.4 B and D show that there is not a significant increase in surviving cells in the populations that have previously survived GSK461364 treatment. This fits with the results of the MTS assay, in suggesting that the colony formation assay has not selected for the survival of a resistant population of cells.

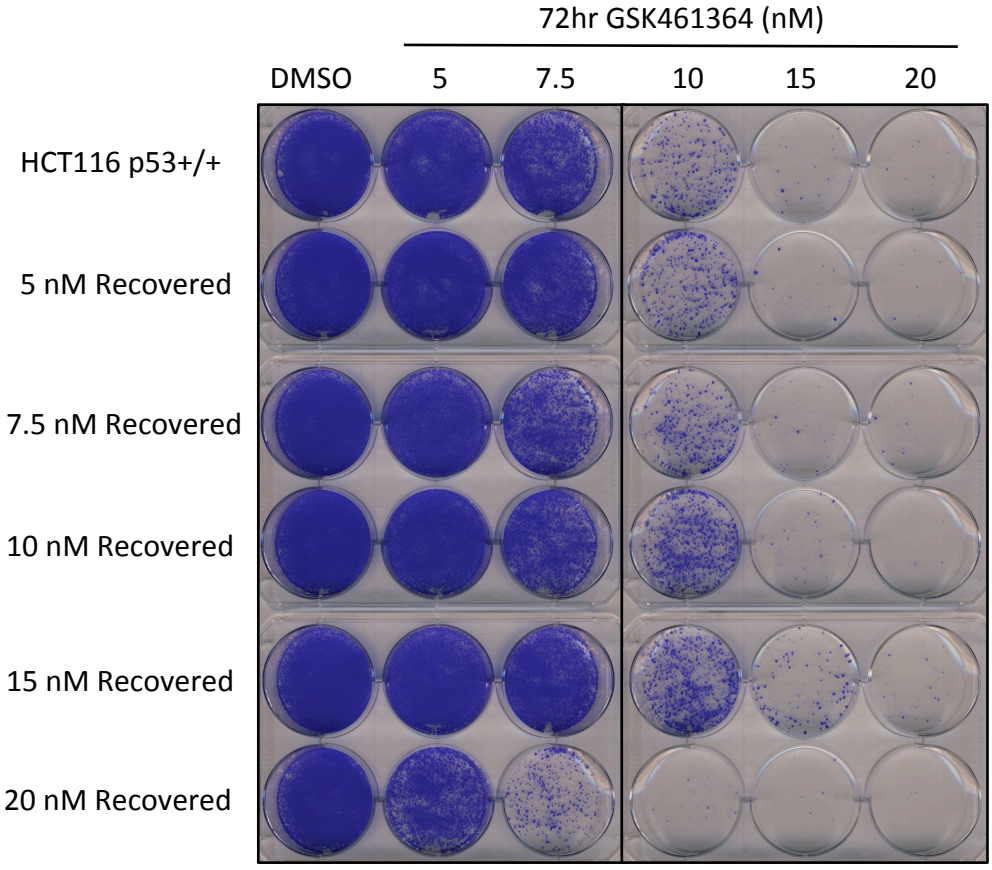
Next, the same procedure was carried out using BI6727. HCT116 p53+/+ and p53-/- cells were treated in the same manner as above, but with BI6727 replacing GSK461364 at each stage. Again, MTS and colony formation assays and were used for the cells recovering from each concentration of BI6727. As can be seen in Figure 3.5 the cells recovering from treatment showed no obvious difference in sensitivity to BI6727 compared to cells that had never been treated in both the MTS and colony formation assays. This suggests that HCT116 p53+/+ and p53-/- cells surviving either GSK461364 or BI6727 are not a resistant sub population of cells within the culture and this is irrespective of the p53 status.

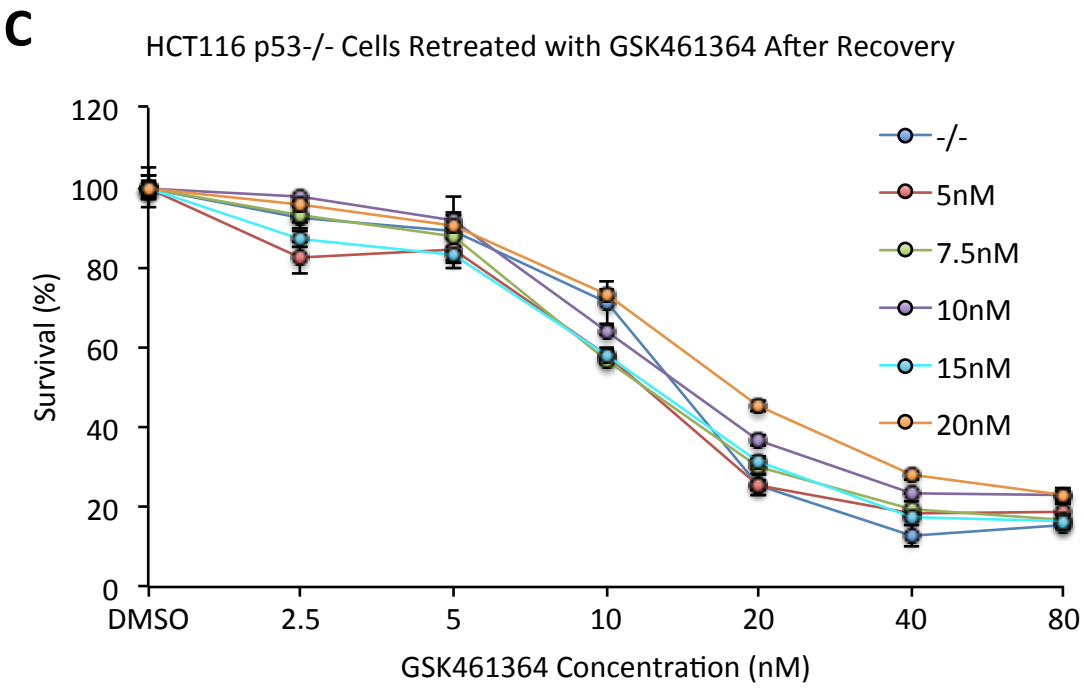
To further enhance this experiment, U2OS cells were then treated in the same way to determine the outcome in a different cellular background. First the U2OS cells were treated with 5, 7.5, 10, 15 or 20 nM GSK461364 or 5, 7.5, 10 or 15 nM BI6727. The cells surviving treatment were then plated for an MTS assay. Little differences were observed between U2OS cells that had never been exposed to PLK1 inhibitors and the cells that had survived treatment as shown in Figure 3.6. Again this supported the result that there is not a population of cells within the culture that are resistant to PLK1 inhibitors and potentially providing surviving cells.

A


HCT116 p53+/+ Cells Retreated with GSK461364 After Recovery
Survival (%)
120
100
80
60
40
20
0
DMSO
2.5
5
10
20
40
80
GSK461364 Concentration (nM)
+/+
5 nM
7.5 nM
10 nM
15 nM
20 nM

B


72hr GSK461364 (nM)
DMSO
5
7.5
10
15
20
HCT116 p53+/+
5 nM Recovered
7.5 nM Recovered
10 nM Recovered
15 nM Recovered
20 nM Recovered



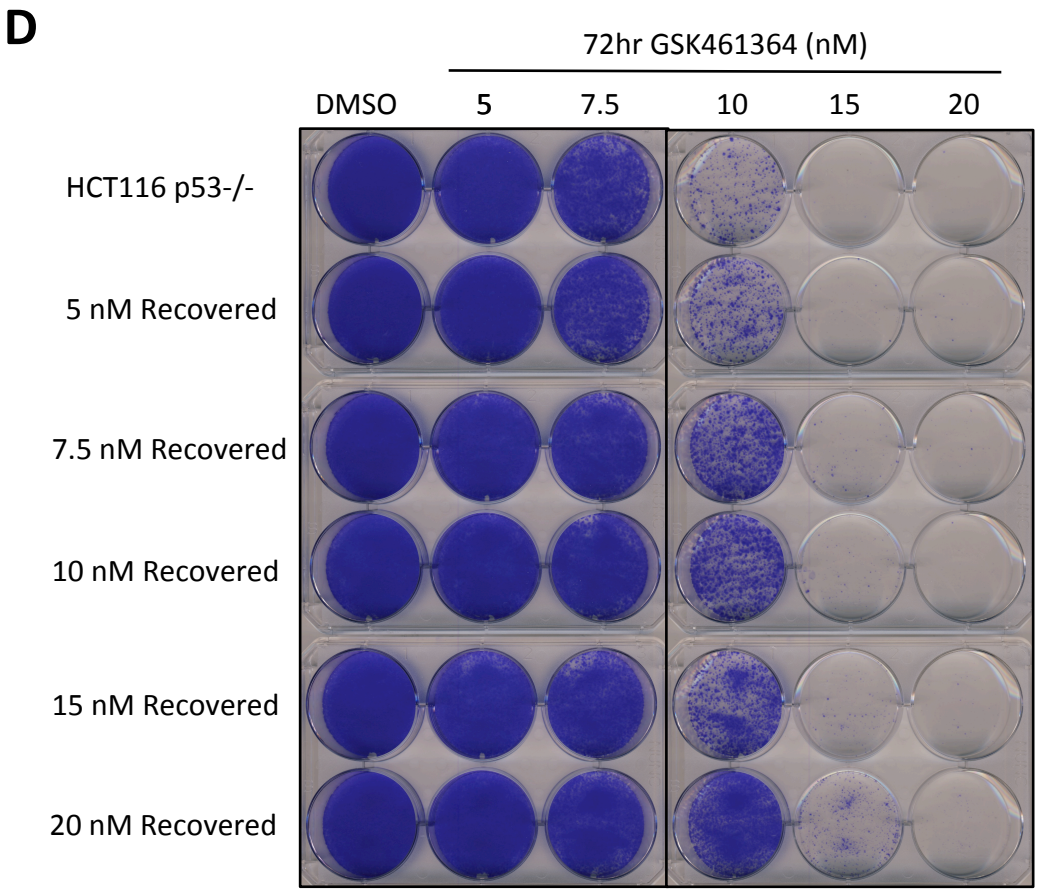
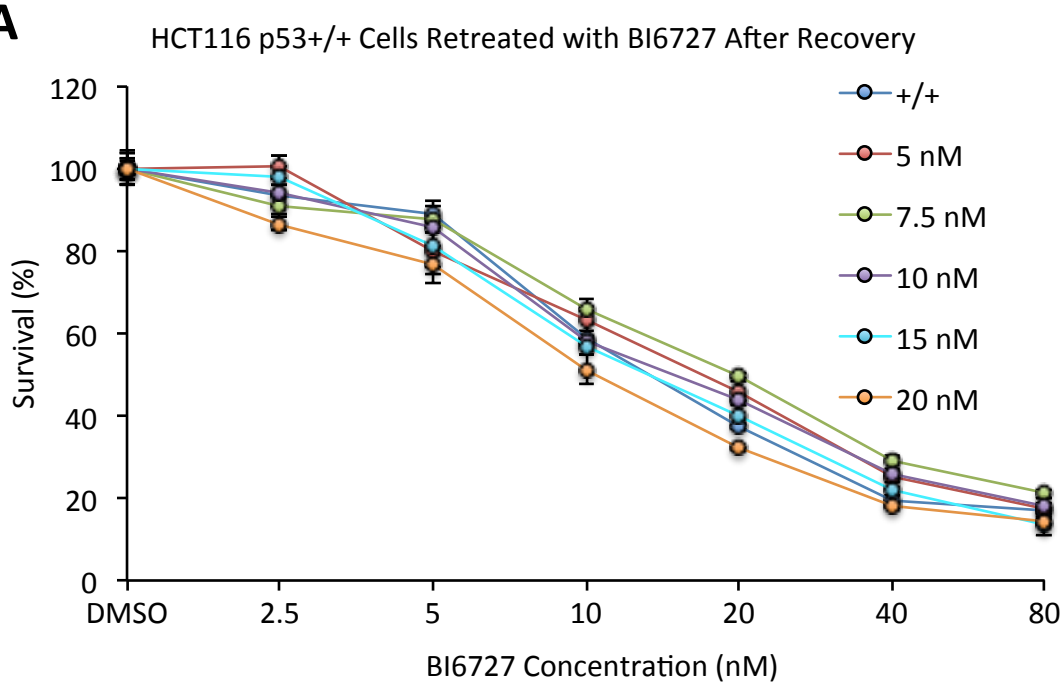
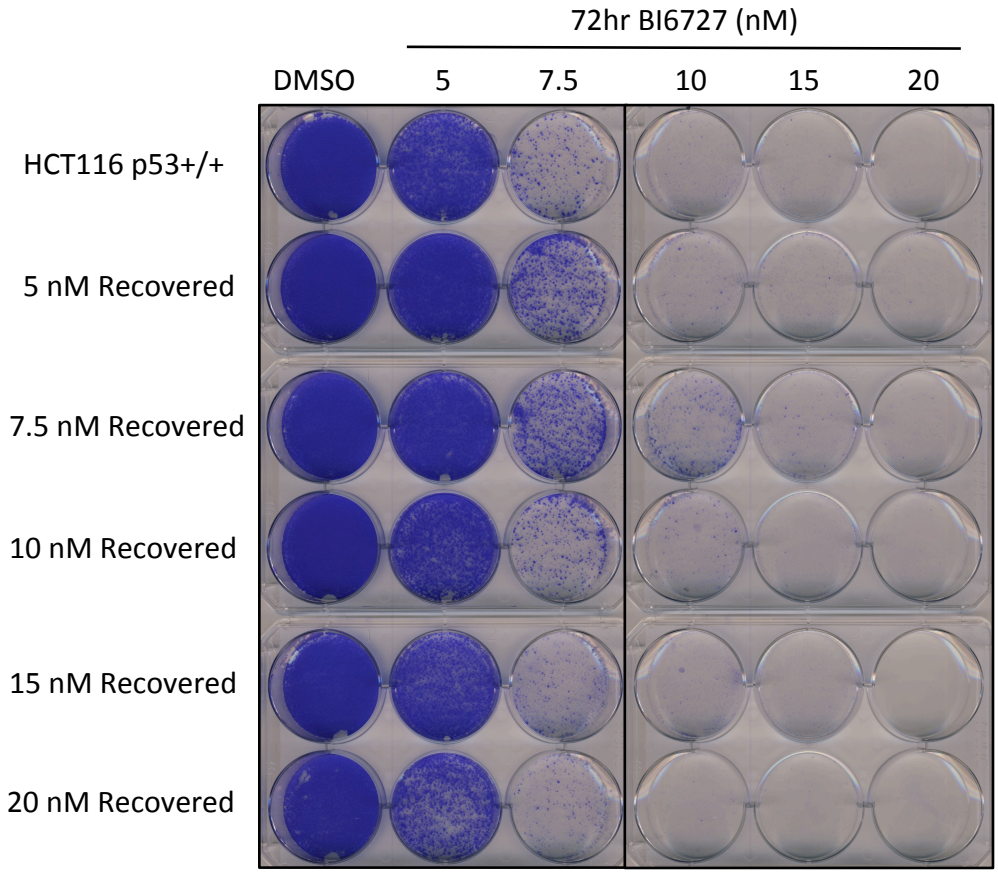


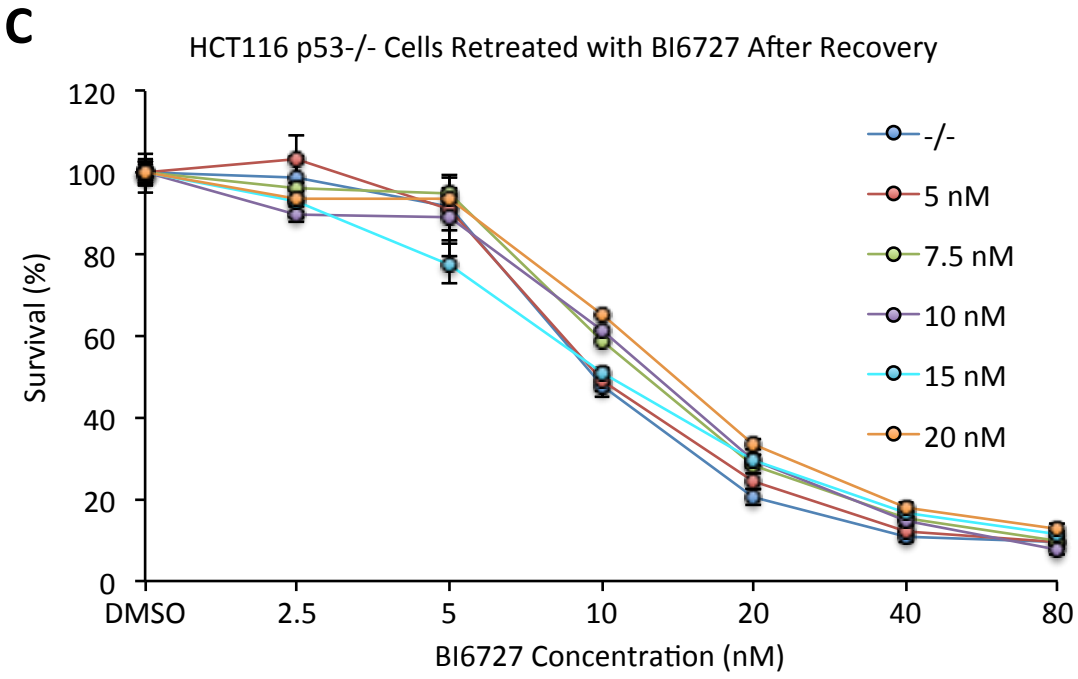
Figure 3.4. Cells surviving treatment with GSK461364 are not a result of a resistant population of cells. HCT116 p53+/+ and p53-/- cells were treated with 5, 7.5, 10, 15 or 20 nM GSK461364 for 72 hours. The drug was then removed and the surviving cells were allowed to recover. When enough cells were obtained they were plated for further experiments along with the parental HCT116 p53+/+ and p53-/- cells that had not been exposed to GSK461364. For MTS assays cells were plated and subsequently treated with 2.5, 5, 10, 20, 40 or 80 nM GSK461364 for a further 72 hours. The resulting survival is shown for HCT116 p53+/+ (A) and p53-/- (C) populations. The graphs are representative of this procedure being carried out twice and each time samples being tested in triplicate. For colony formation assays cells were plated and treated for 72 hours with DMSO, 5, 7.5, 10, 15 or 20 nM GSK461364. The drug was then removed and the cells were allowed to recover in fresh media for a further 10 days before fixing and staining with crystal violet. Survival of HCT116 p53+/+ (B) and p53-/- (D) cells is shown.

A


HCT116 p53+/+ Cells Retreated with BI6727 After Recovery
Survival (%)
120
100
80
60
40
20
0
DMSO
2.5
5
10
20
40
80
BI6727 Concentration (nM)
+/+
5 nM
7.5 nM
10 nM
15 nM
20 nM

B


72hr BI6727 (nM)
DMSO
5
7.5
10
15
20
HCT116 p53+/+
5 nM Recovered
7.5 nM Recovered
10 nM Recovered
15 nM Recovered
20 nM Recovered



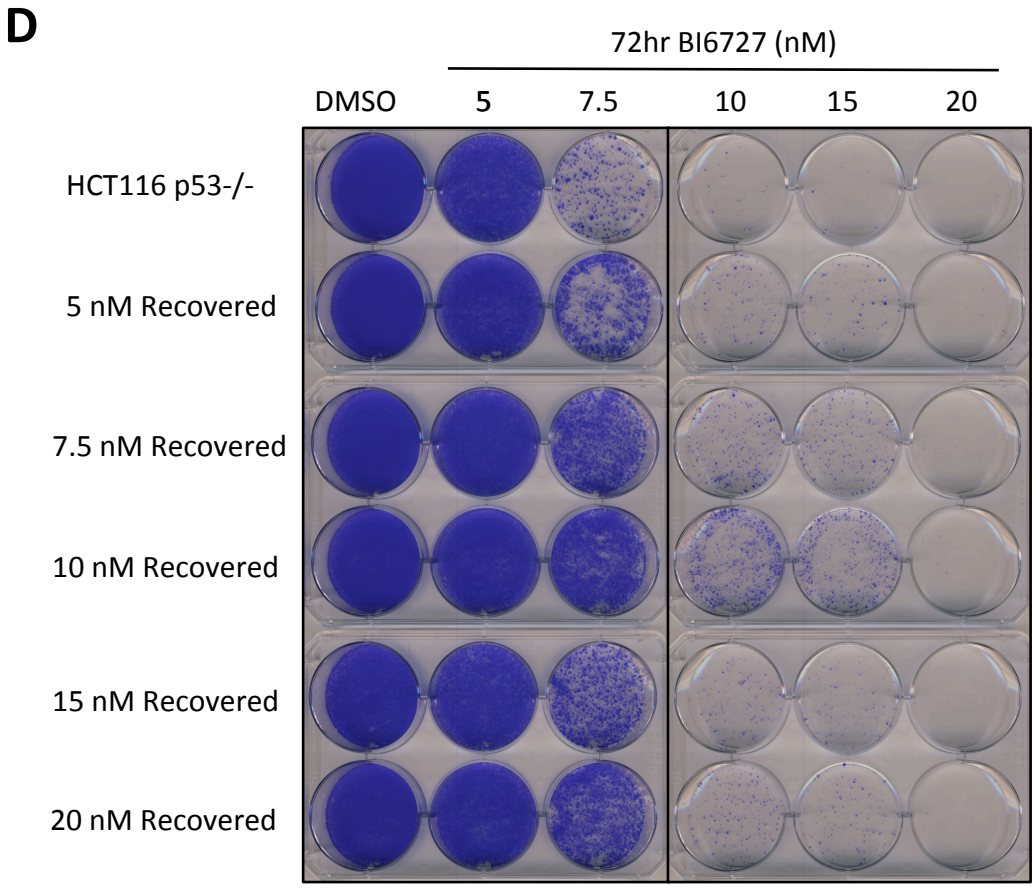


Figure 3.5. Cells surviving treatment with BI6727 are not a result of a resistant population of cells. HCT116 p53+/+ and p53-/- cells were treated with 5, 7.5, 10, 15 or 20 nM BI6727 for 72 hours. The drug was then removed and the surviving cells were allowed to recover. When enough cells were obtained they were plated for further experiments along with the parental HCT116 p53+/+ and p53-/- cells that had not been exposed to BI6727. For MTS assays cells were plated and subsequently treated with 2.5, 5, 10, 20, 40 or 80 nM BI6727 for a further 72 hours. The resulting survival is shown for HCT116 p53+/+ (A) and p53-/- (C) populations. The graphs are representative of this procedure being carried out twice and each time samples being tested in triplicate. For colony formation assays cells were plated and treated for 72 hours with DMSO, 5, 7.5, 10, 15 or 20 nM BI6727. The drug was then removed and the cells were allowed to recover in fresh media for a further 10 days before fixing and staining with crystal violet. Survival of HCT116 p53+/+ (B) and p53-/- (D) cells is shown.

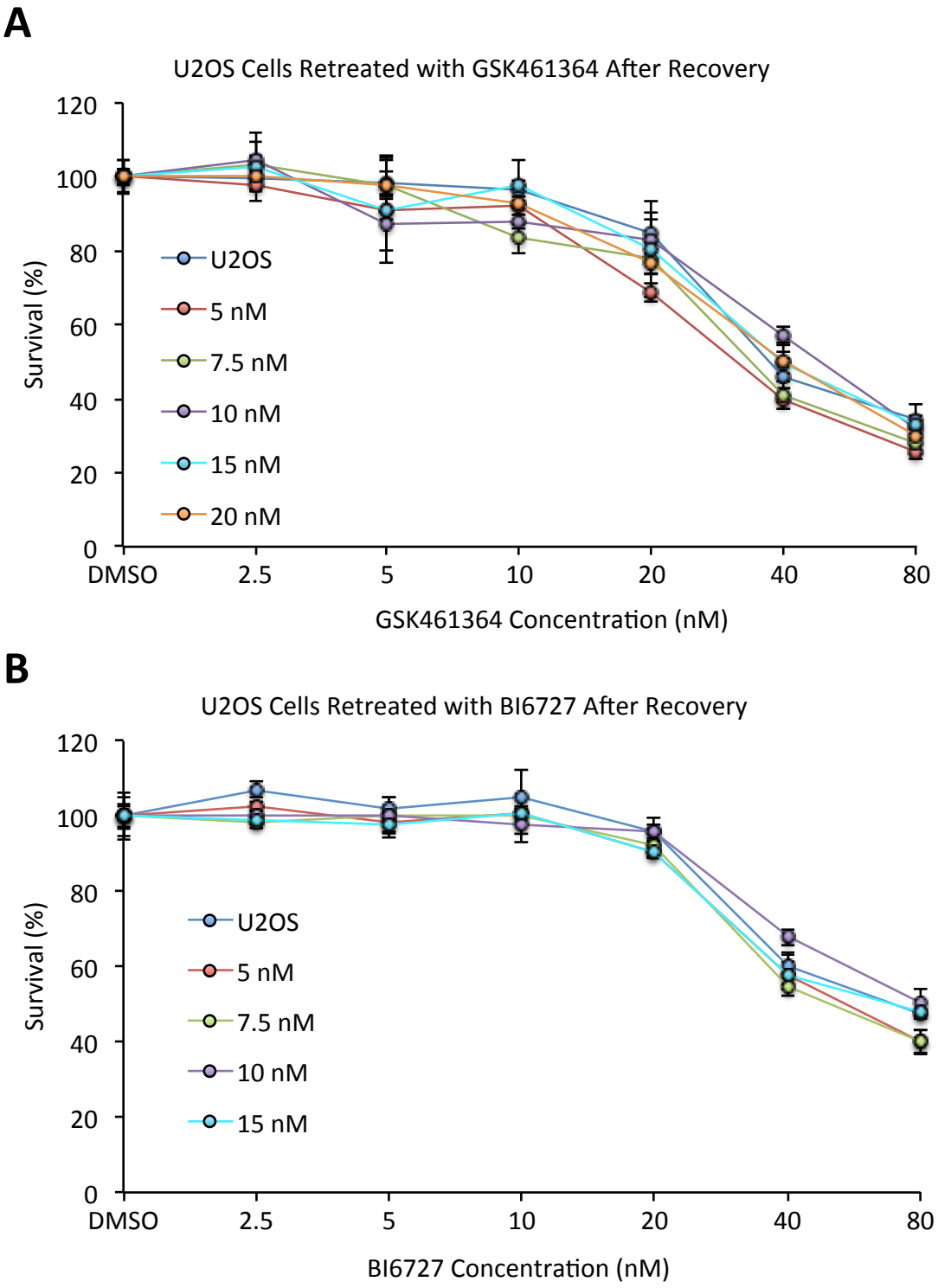


Figure 3.6. U2OS cells surviving treatment with GSK461364 or BI6727 are not a result of a resistant population of cells. U2OS cells were treated with 5, 7.5, 10, 15 or 20 nM GSK461364 or 5, 7.5, 10 or 15 nM BI6727 for 72 hours. The drug was then removed and the surviving cells were allowed to recover. When enough cells were obtained they were plated for MTS assays along with parental U2OS cells that had not been exposed to PLK1 inhibitors. Plated cells were subsequently treated with 2.5, 5, 10, 20, 40 or 80 nM GSK461364 (A) or BI6727 (B) for a further 72 hours. Cell survival was then determined by a MTS assay. The graphs are representative of this procedure being carried out twice and each time samples being tested in triplicate.

3.4. Discussion

The findings of this chapter support previous observations that p53 reduces sensitivity to PLK1 inhibitors (Guan *et al.*, 2005; Liu, Lei and Erikson, 2006; Sur *et al.*, 2009; Degenhardt *et al.*, 2010; McKenzie *et al.*, 2010; Danovi *et al.*, 2013; Yim and Erikson, 2014). By using the HCT116 isogenic lines, the effect of p53 could be focused upon, as the p53 status is the only difference between these lines. Both a short term survival assay and longer term recovery assay had similar findings in regards to the p53 status. Additionally, further experiments showed that the surviving colonies were not a selection of resistant clones in the population, therefore the protective effect of p53 must occur through an innate mechanism. Interestingly, the effects of Taxol were p53 independent in these assays. This suggested that p53 does not have the ability to alter sensitivity to all mitotic inhibitors. Previous studies have shown that the sensitivity of different cancer cells and patient responses to Taxol are independent of p53, supporting the findings in this thesis (Debernardis *et al.*, 1997; Rantanen *et al.*, 2002; Reinecke *et al.*, 2005; Sezgin *et al.*, 2005).

There is evidence to suggest that PLK1 upregulation in cancer is associated with mutations in p53 (King *et al.*, 2012; Watanabe *et al.*, 2015), and the clinical outcome for these patients is poorer than cancers expressing PLK1 or *TP53* mutations alone (King *et al.*, 2012). This would imply that treatment of patients that express PLK1 and lack functional p53 may be ideal candidates for treatment with PLK1 inhibitors. Whilst numerous clinical trials have been carried out using PLK1 inhibitors, and the patient response varies considerably within each study, to date, these trials have not investigated whether the p53 status of patients could be a reason for the differences in response to PLK1 inhibitors. This would be an interesting study to conduct in the future and could enhance the clinical efficacy of PLK1 inhibitors by targeting patients most likely to respond to this type of treatment.

Chapter 4 : PLK1 inhibitors lead to induction of p53 through the DNA damage response pathways

4.1. Background

It has been shown in chapter 3, and by others, that the presence of p53 results in a protective effect when PLK1 inhibitors are applied. However, it has not been shown how this may occur. Previous studies have shown that PLK1 inhibitors lead to an arrest in early mitosis, at the prometaphase stage. Upon treatment with PLK1 inhibitors, cells generally accumulate in mitosis with monopolar spindles, due to a failure in centrosome separation. This forms the classic 'polo' effect of the condensed chromosomes. Some studies have suggested that inhibition of PLK1 can also lead to an intra-S-phase arrest (Mandal and Strebhardt, 2013). Additionally, inhibition of PLK1 has been shown to result in DNA damage and apoptosis. However, how these observations link to p53 and the p53 status of cancer cells is an area of research that is currently uncovered.

One of the classic activators of p53 is DNA damage. Upon DNA damage, p53 is phosphorylated by ATM and ATR at serine 15. This phosphorylation is critical for p53 activation and function in transactivating downstream targets, such as p21 (Loughery *et al.*, 2014). The functional effects of activated p53 can vary depending on the input stress type and strength. In general, there are two outcomes, cell cycle arrest (either temporary to allow repair to occur or a permanent total growth arrest) or cell death (generally through apoptosis). Therefore, p53 can have dramatic effects on the cell cycle. Flow cytometry is a useful technique that can be used to assess the cell cycle distribution of a population of cells, allowing cell cycle arrests to be observed. This technique is therefore highly useful for investigating p53 dependent effects.

It is well established that p53 responds to failed mitoses. When a cell enters G1 with an abnormal DNA complement, due to mitotic failure, p53 facilitates cell cycle arrest or apoptotic cell death to prevent genomic instability. Additionally, if DNA damage occurs during mitosis, it has been suggested that p53 is alerted to this damage upon mitotic exit

(Hayashi and Karlseder, 2013). During mitosis the DNA damage response is limited. It has been suggested that a prolonged mitotic arrest can lead to the formation of DNA damage foci at the telomeres. This DNA damage response at the telomeres is thought to be caspase dependent (Hain *et al.*, 2016). The partially deprotected telomeres are recognised by p53 when the cell exits mitosis and enters G1 and such cells generally undergo apoptotic cell death. There are therefore numerous potential ways in which p53 may respond to PLK1 inhibition.

This chapter makes use of the HCT116 p53+/+ and p53-/- isogenic lines once again. However, in order to ensure that any observed effects are due to p53, an alternative approach of looking specifically at p53 function was utilised. By using siRNA, targeting p53, p53 could be silenced to allow investigation of p53-dependent effects in different established cell lines. Some experiments have also been carried out using MCF-7 cells, in which both the parental MCF-7 p53 wild type cells and MCF-7 cells that have had p53 knocked out by CRISPR technology have been tested. These cells were a kind gift from Professor Laki Buluwela. With the range of cell lines available, flow cytometry was used to allow analysis of cell cycle distribution and western blotting for determination of differences at the protein level of proteins of interest.

4.2. Aims

The aims of this chapter are to determine how p53 may offer a protective effect to cancer cells treated with PLK1 inhibitors. To do this the p53-dependent cell cycle distribution differences upon treatment with PLK1 inhibitors was investigated and the effects of PLK1 inhibitors on p53 and why they may have such effects was researched.

4.3. Results

4.3.1. Treatment with PLK1 inhibitors results in a p53 dependent G1 peak

To try and address the reasons for p53 dependent differences in sensitivity to PLK1 inhibitors, the aim was to determine if there were any obvious differences between the HCT116 p53+/+ and p53-/- cells upon treatment with PLK1 inhibitors. To initially address this, flow cytometry was used to assess the cell cycle distribution. Prior to harvesting, BrdU was added to the samples to allow representation of cells in S phase. BrdU is a thymidine analogue that is incorporated into the DNA during DNA synthesis. Once fixed, cells were incubated with an anti-BrdU antibody, FITC secondary antibody and finally labelled with propidium iodide to discriminate between differences in DNA content. Propidium iodide intercalates into the DNA and allows the stage of the cell cycle to be assessed, due to cells in G2/M containing double the DNA content compared to G1, and therefore double the propidium iodide.

The HCT116 p53+/+ and p53-/- cells were seeded in 6 cm plates, and treated the following day with DMSO (vehicle control), 20 nM GSK461364 or 10 nM BI6727. After staining, cells were analysed on the flow cytometer and the resulting profiles are shown in Figure 4.1. As can be seen in Figure 4.1 the DMSO treated samples had similar cell cycle distributions between the p53+/+ and p53-/- cells. Upon treatment with either PLK1 inhibitor, the major effect is the presence of a sharp peak at 100K, representing a G2/M arrest, in both the p53+/+ and p53-/- lines. In both lines, a peak of <2N is present, representing cell death, and also >4N representing endoreduplication/multi or micro nucleation. Whilst these are fairly similar between the two lines, the one notable difference observed was the presence of a 2N peak, representing cells in G1, in the p53+/+ line that was absent from the p53-/- line.

Whilst the two HCT116 lines are isogenic, during culture of cancer cells, changes can occur. To confirm whether the PLK1 inhibitor induced G1 cells are dependent on wild type p53, or if this could be a cell line specific outcome, p53 was silenced by siRNA in the wild type HCT116 line. siRNAs target specific mRNAs for degradation resulting in gene silencing. A non-silencing siRNA or siRNA targeting p53 were used to mimic the HCT116 p53+/+ and p53-/- lines respectively. After 24 hours of silencing the cells were harvested and western blotting was used to determine the protein levels. As can be seen in Figure 4.2 the p53 siRNA is effective, as the p53 levels are lower than the non-silencing control. This showed that 24 hours of knockdown was a suitable time to carry out further treatments. HCT116 cells were then transfected with non-silencing siRNA or sip53 for 24 hours before treatment with PLK1 inhibitors for a further 24 hours. Flow cytometry was then used to determine the cell cycle distribution. The knockdown of p53 had little effect on the cell cycle distribution of cells treated with the DMSO control. In the presence of PLK1 inhibitors, a G2/M arrest was observed in both the non-silencing and p53 silenced samples. However, as with the HCT116 p53+/+ and p53-/- lines the G1 peak was present in the p53 wild type (non-silencing) and whilst not absent, was much smaller in the p53 knockdown samples. Although in the HCT116 p53-/- cells the G1 peak was abolished and in the p53 siRNA treated samples it was only reduced, this could be explained by the fact that siRNA only results in knockdown and not knockout, therefore the residual p53 may be enough to cause an effect.

To determine if this effect was an artefact of the HCT116 lines, U2OS (a p53 wild type osteosarcoma line) and MCF-7 (a p53 wild type breast cancer line) were also used in the same experiment. In order to allow a similar comparison to the HCT116 p53+/+ and p53-/- cells siRNA targeting p53 was again used. Figure 4.3 shows successful knockdown of p53 in U2OS cells after 24 hours. Actin has been probed to show even loading of protein samples in each well. U2OS cells were therefore treated with non-

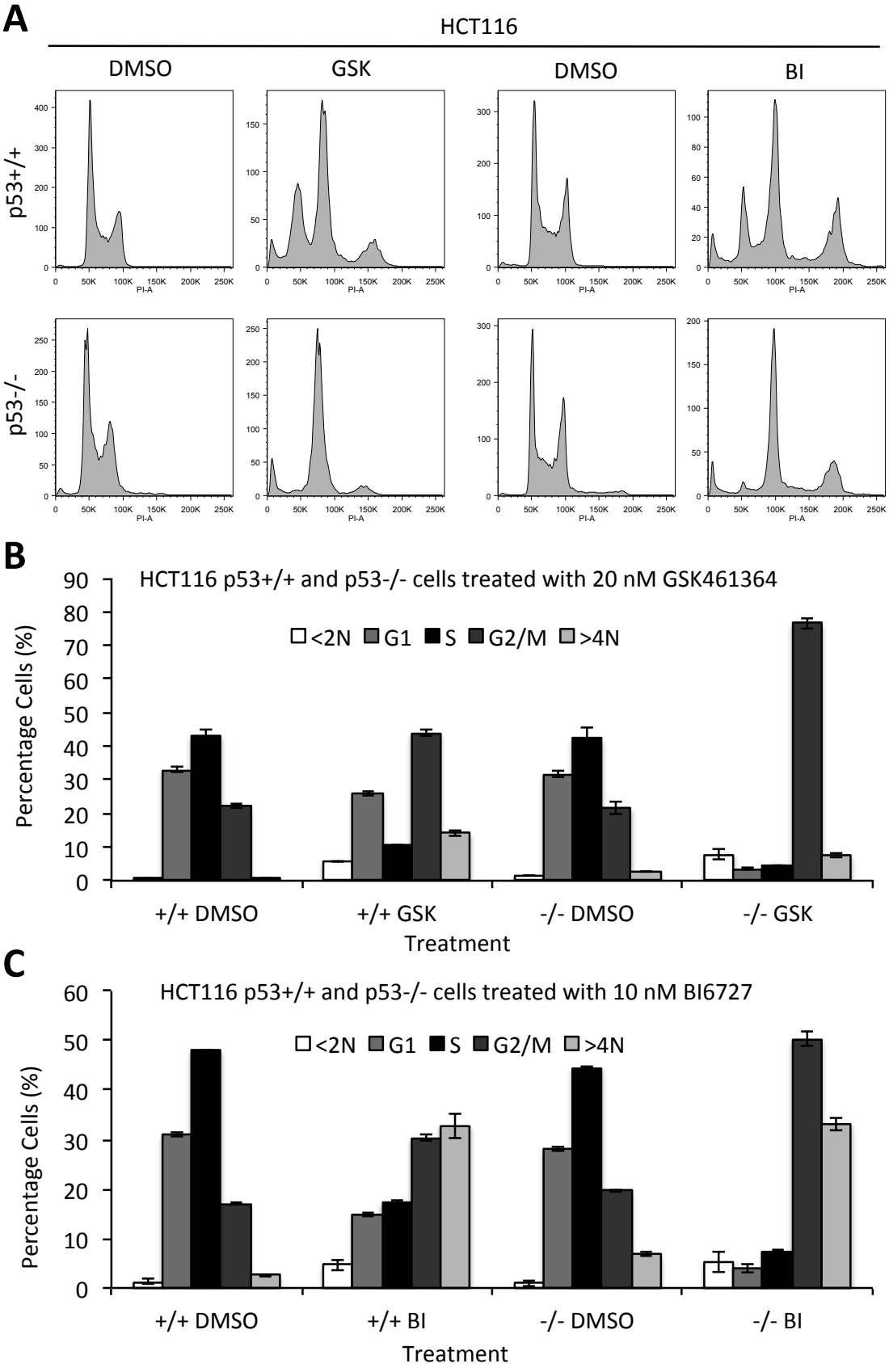


Figure 4.1. Cells expressing wild type p53 show a partial G1 arrest in response to treatment with PLK1 inhibitors, GSK461364 or BI6727. HCT116 p53+/+ and p53-/- cells were treated for 24 hours with 20 nM GSK461364, 10 nM BI6727 or with DMSO as control. The cells were subsequently harvested and analysed by flow cytometry, with the resulting histograms shown (A) and quantification of this data for GSK461364 (B) and BI6727 (C). The data are representative of two independent experiments each conducted in triplicate.

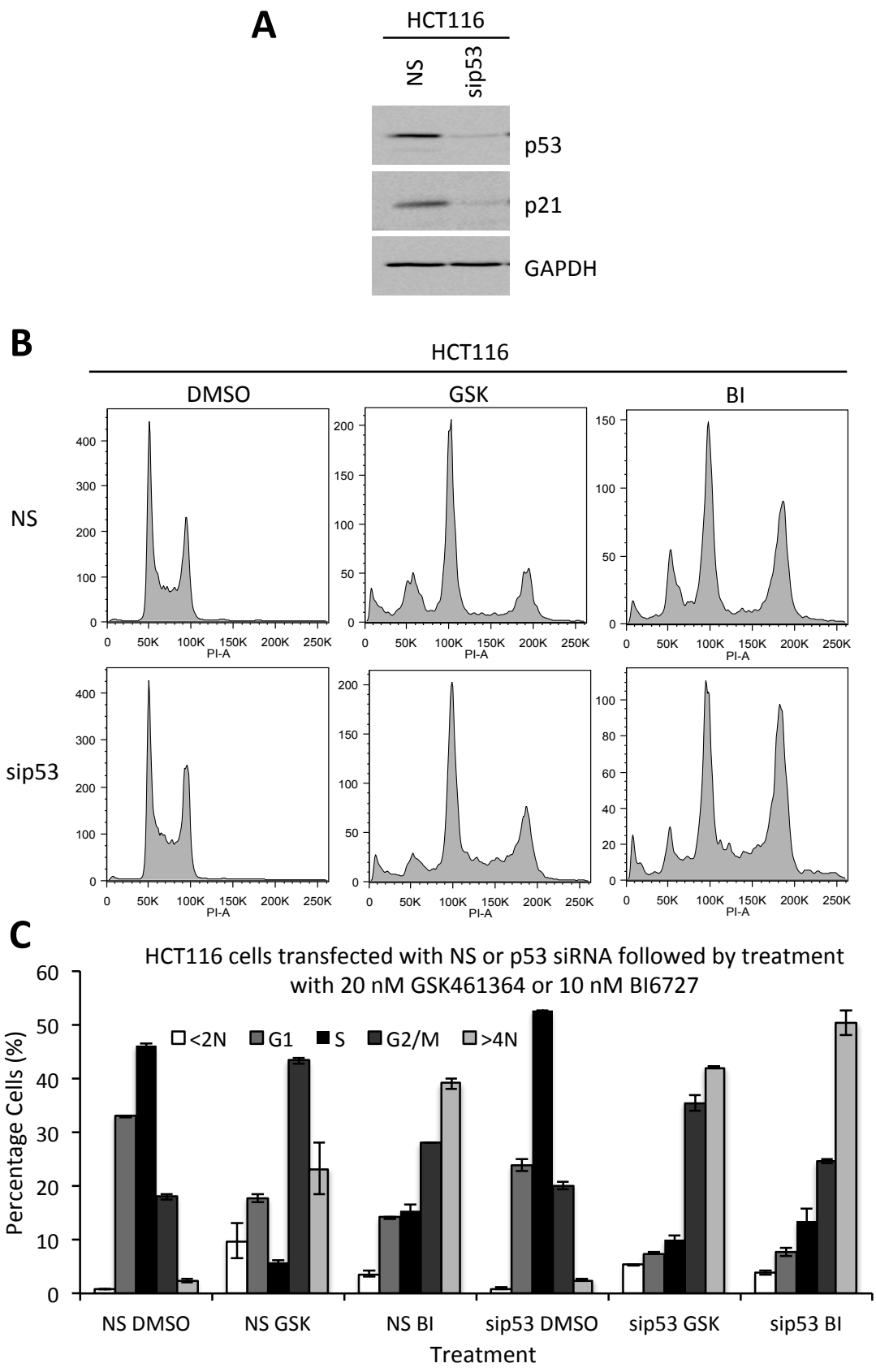


Figure 4.2. Partial G1 arrest of HCT116 cells in response to treatment with the PLK1 inhibitors, GSK461364 or BI6727, is dependent upon p53. HCT116 cells were transfected with non-silencing siRNA or siRNA targeting p53 for 24 hours, before addition of 20 nM GSK461364, 10 nM BI6727 or DMSO for a further 24 hours. Cells were harvested for western blotting (A) or flow cytometry, with the resulting histograms shown in (B) and quantification of this data in (C). The data are representative of two independent experiments each conducted in duplicate.

silencing or p53 siRNA for 24 hours, before addition of GSK461364 (20 nM) or BI6727 (10 nM) for a further 24 hours. Cells were then harvested and flow cytometry was again used to assess the cell cycle distribution. As can be seen in figure 4.3 the sip53 caused a slight change in the cell cycle profile compared to the non-silencing siRNA with DMSO treatment. Silencing p53 in these cells resulted in an increase in cell death. However, even with the differences observed under control conditions, when treated with PLK1 inhibitors the trend of the cell cycle distribution was very similar to that observed in the HCT116 p53+/+ and p53-/- cells. With the GSK461364 drug the sip53 sample had a complete removal of the G1 peak compared to the non-silencing control, and the BI6727 drug showed a reduction of the G1 peak with silencing of p53.

MCF-7 cells were then treated in the same way as U2OS cells. The knockdown after 24 hours was effective, with loss of the p53 band as shown in the western blot in Figure 4.4. Flow cytometry was then used. Figure 4.4 shows that the DMSO controls for MCF-7 cells were similar with either the non-silencing or p53 siRNA. Upon treatment with PLK1 inhibitors the profiles were similar to that of HCT116 cells, with the predominant effect being the accumulation of cells in G2/M. Like the HCT116 p53+/+ cells, the non-silencing control had a G1 peak. The p53 siRNA samples also had a G1 peak present, but this peak was smaller than that of the non-silencing control.

These results show that cells expressing wild type p53 (HCT116 p53+/+ or non-silencing HCT116, U2OS or MCF-7 cells) give rise to a higher proportion of cells in G1 upon treatment with PLK1 inhibitors than in the absence of p53 (HCT116 p53-/- or sip53 in HCT116, U2OS or MCF-7 cells). Since p53 was shown to offer a protective effect upon inhibition of PLK1, and the presence of the G1 peak is the only noticeable difference between absence and presence of p53, this seems the most plausible explanation for the source of protection of p53 competent cancer cells.

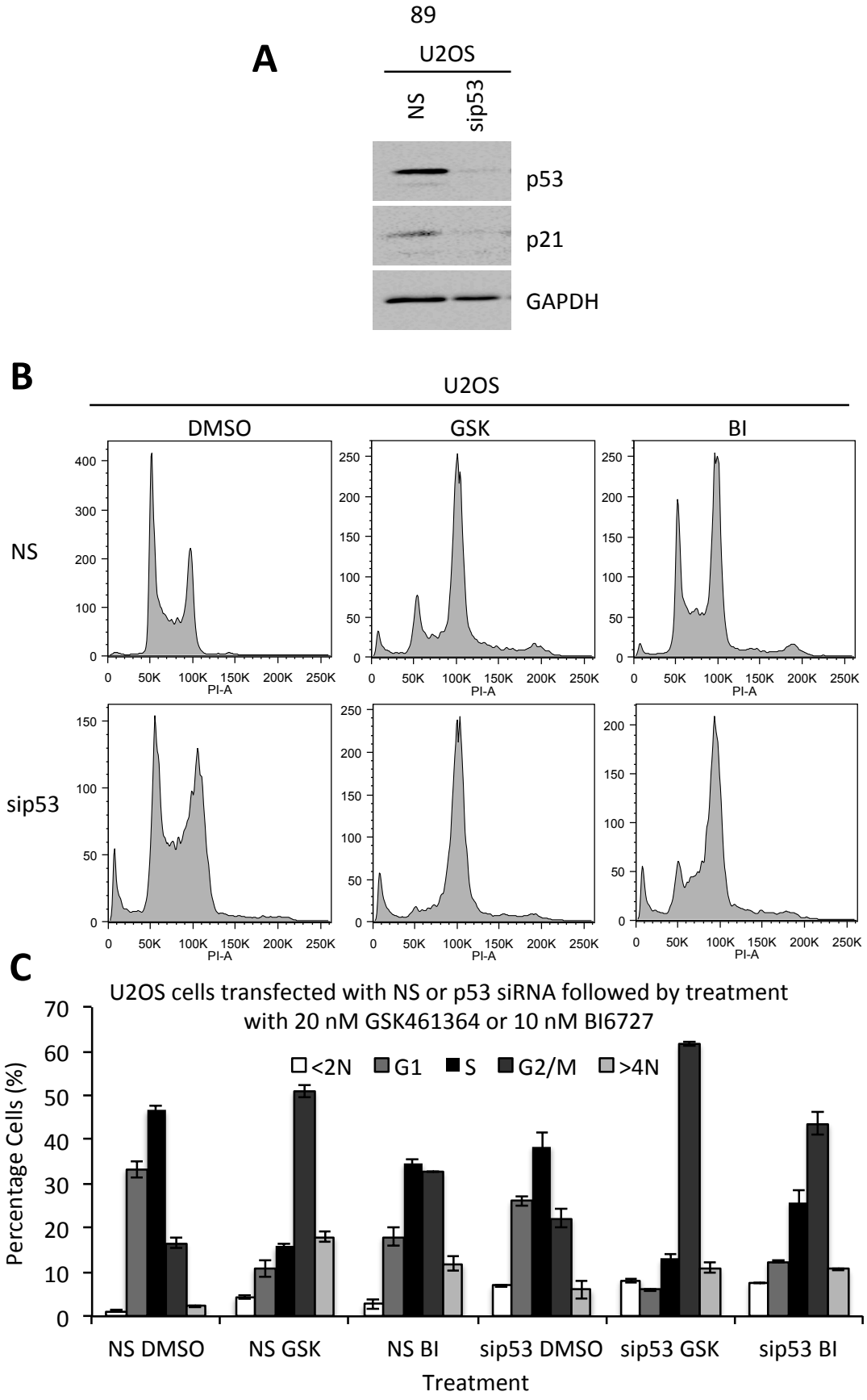


Figure 4.3. Partial G1 arrest of U2OS cells in response to treatment with the PLK1 inhibitors, GSK461364 or BI6727, is dependent upon p53. U2OS cells were transfected with non-silencing siRNA or siRNA targeting p53 for 24 hours, before addition of 20 nM GSK461364, 10 nM BI6727 or DMSO for a further 24 hours. Cells were harvested for western blotting (A) or flow cytometry, with the resulting histograms shown in (B) and quantification of this data in (C). The data are representative of two independent experiments each conducted in duplicate.

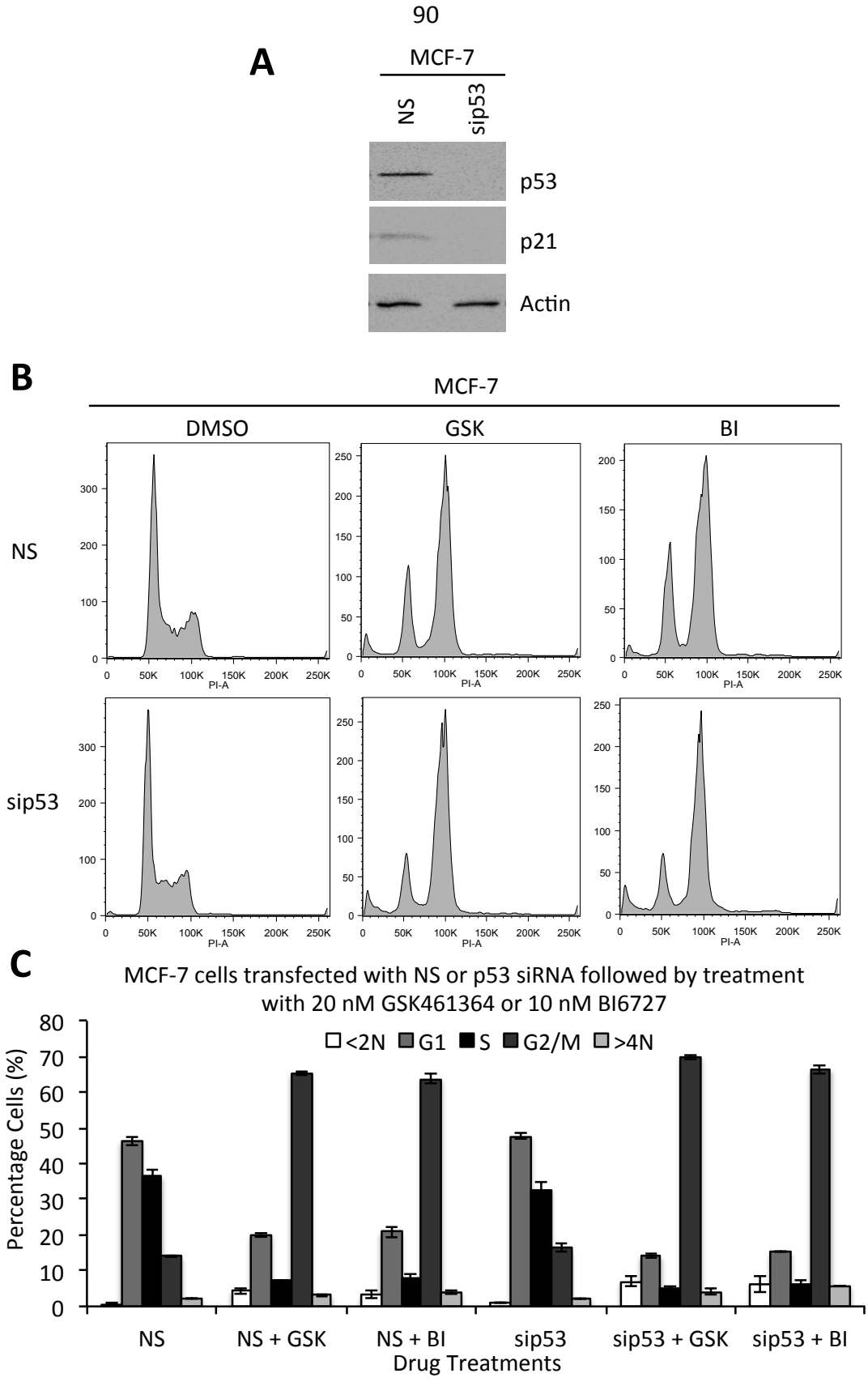


Figure 4.4. Partial G1 arrest of MCF-7 cells in response to treatment with the PLK1 inhibitors, GSK461364 or BI6727, is dependent upon p53. MCF-7 cells were transfected with non-silencing siRNA or siRNA targeting p53 for 24 hours, before addition of 20 nM GSK461364, 10 nM BI6727 or DMSO for a further 24 hours. Cells were harvested for western blotting (A) or flow cytometry, with the resulting histograms shown in (B) and quantification of this data in (C). The data are representative of two independent experiments each conducted in duplicate.

4.3.2. PLK1 inhibitors lead to induction of p53

The next aim was to determine what was occurring at the protein level in order to better understand the involvement of p53. HCT116 p53+/+ and p53-/- cells were again seeded in 6 cm plates, and were untreated or treated with DMSO, 20 nM GSK461364 or 10 nM BI6727. As the flow cytometry experiments were carried out after 24 hours of treatment, this time point was again selected. However, to gain insight into longer-term effects, 48 and 72 hours of treatment were also tested for this experiment. The cells were therefore harvested after 24, 48 or 72 hours of treatment and western blotting was used to determine protein levels. As can be seen in Figure 4.5 the PLK1 levels increase at 24 hours of treatment compared to the untreated and DMSO treated controls. As PLK1 inhibitors only inhibit the activity of PLK1 and do not repress the expression of PLK1, this is expected as the cells arrest in mitosis where PLK1 is expressed at its highest levels (Golsteyn *et al.*, 1994). At 48 and 72 hours the PLK1 levels are again reduced, presumably due to the transient nature of the mitotic arrest, resulting in cells eventually continuing through to exit or abort mitosis. Next, looking at the p53 levels showed that p53 was increasing upon treatment with PLK1 inhibitors. Correspondingly, p21, a classic p53 downstream target was also seen to increase. Phosphorylated gamma H2AX (S139) (γ-H2AX), a marker of DNA damage (specifically double strand breaks) was seen to increase, along with cleaved PARP, a marker of apoptosis. Interestingly, in the HCT116 p53+/+ line the level of γ-H2AX is lower upon treatment with PLK1 inhibitors in comparison to the HCT116 p53-/- cells. Finally, phosphorylated histone H3 at serine 10, a marker of mitosis, mirrored the PLK1 levels, suggesting cells accumulate at mitosis by 24 hours, and the cells subsequently exit mitosis at 48-72 hours. As a loading control, actin has also been probed, to show even loading of protein samples in each well.

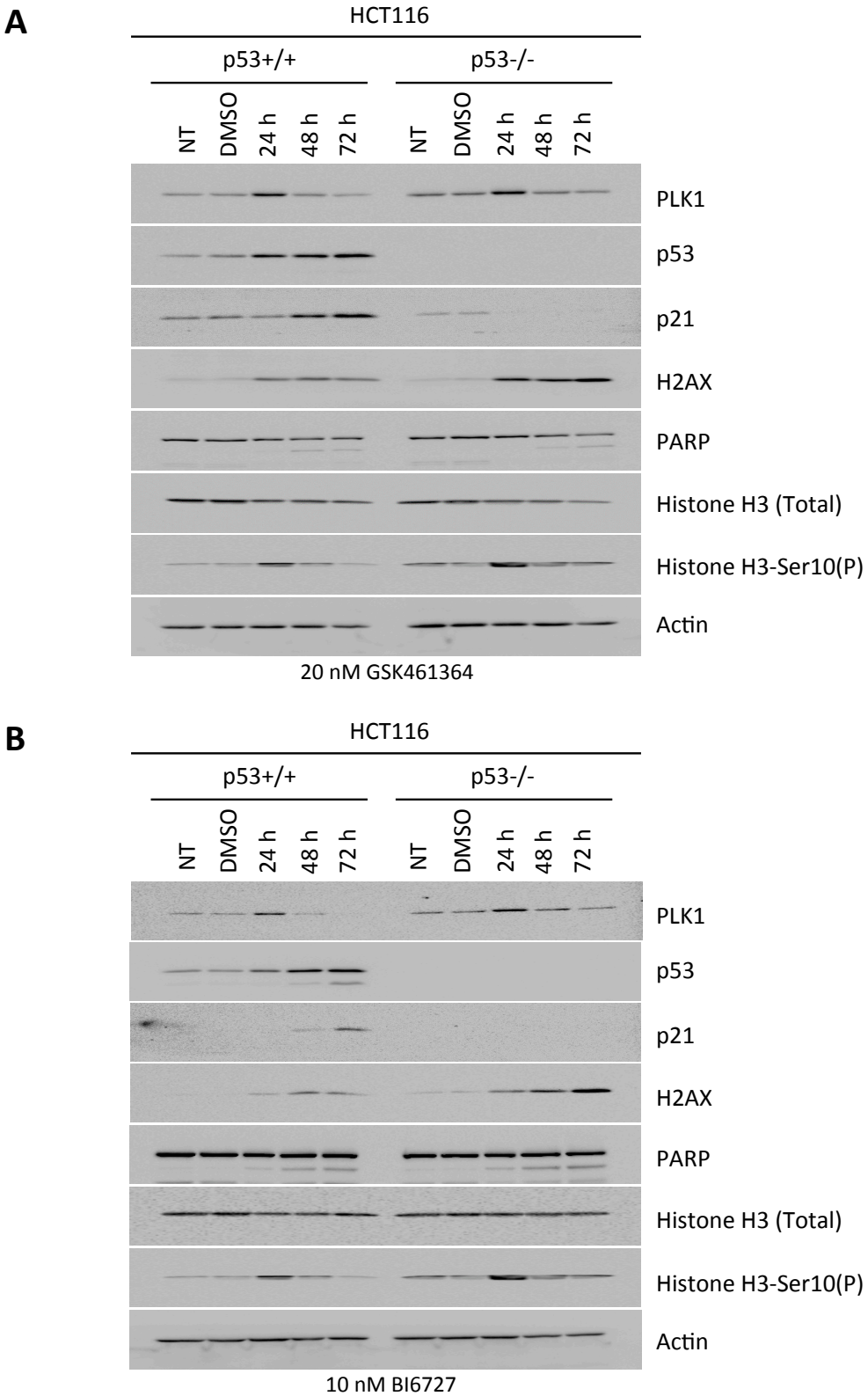


Figure 4.5. Treatment of HCT116 cells with PLK1 inhibitors induces a p53 response. HCT116 p53+/+ and p53-/- cells were either untreated, treated with DMSO or treated for 24, 48 or 72 hours with 20 nM GSK461364 (A) or 10 nM BI6727 (B). Western blotting was then used with the antibodies indicated in the figure.

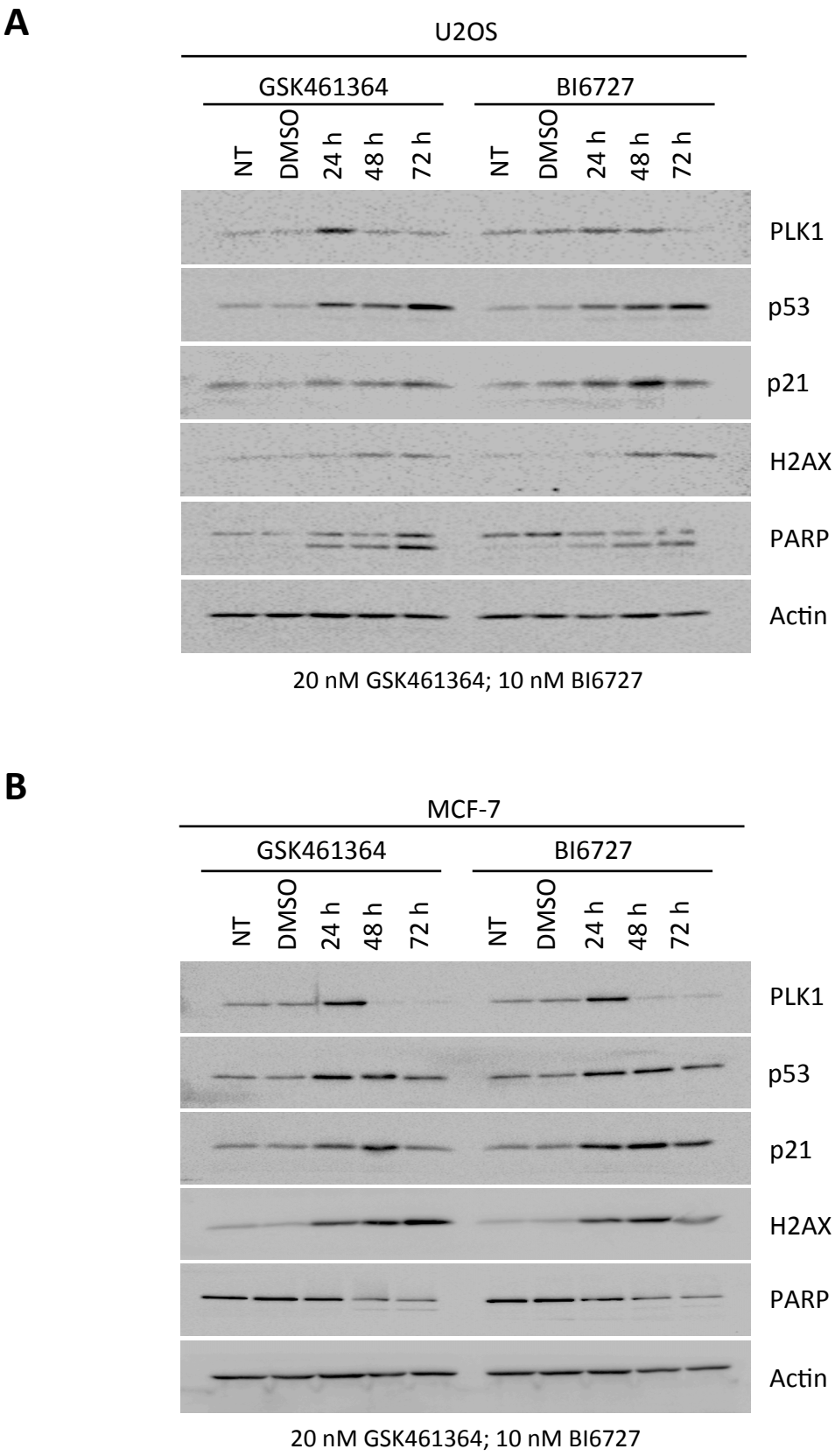


Figure 4.6. Treatment with PLK1 inhibitors induces p53. U2OS (A) and MCF-7 (B) cells were untreated, treated with 20 nM GSK461364 or 10 nM BI6727 for 24, 48 or 72 hours or DMSO as a vehicle control. Western blotting was then carried out with the antibodies indicated in the figure.

Again, to check if these results were specific to the HCT116 lines, MCF-7 and U2OS lines were also tested. In the first instance, MCF-7 and U2OS cells were untreated or treated with DMSO, 20 nM GSK461634 or 10 nM BI6727 for 24, 48 and 72 hours. Western blotting was again used to detect protein levels. Figure 4.6 shows the changes in the protein levels in MCF-7 and U2OS cells treated with both drugs. A similar result to HCT116 cells is observed with both these cell lines, with increasing levels of p53, p21, γ-H2AX and PARP cleavage upon treatment with both of the PLK1 inhibitors. The next experiment aimed to look at the role of p53 in this outcome, so HCT116 and U2OS cells were treated with either non-silencing siRNA or siRNA targeting p53. Figure 4.7 shows the HCT116 cells. The p53 silencing was effective as can be seen from the much lower levels of p53, however, in both the non-silencing and the p53 knockdown samples the PLK1 levels peak at 24 hours of treatment and reduce at 48 and 72 hours. This suggested that p53 is not affecting the levels of PLK1 upon treatment with PLK1 inhibitors. The same approach was used in the U2OS cells, and again the p53 knockdown was successful as can be seen in Figure 4.8. A similar effect was observed in the U2OS cells in regards to the PLK1 levels, with the changes in levels throughout the treatment time being independent of p53. To further investigate the role of p53, parental MCF7 cells, and a derivative line in which p53 has been deleted using CRISPR (a kind gift from Professor Laki Buluwela), were treated with the PLK1 inhibitors. Figure 4.9 shows the changes in protein levels were very similar to that of the HCT116 p53+/+ and p53-/- cells. In the parental line, p53 and p21 levels increase with increasing duration of PLK1 inhibition. In the p53 knock out MCF-7 line, as with the HCT116 p53-/- line, the γ-H2AX levels were higher upon treatment with PLK1 inhibitors compared to the p53 wild type counterpart. The effects of PLK1 inhibitors therefore seem consistent between different cell lines.

A shorter time course was also used, with treatment times of 0, 1, 2, 4, 6, 12 and 24 hours, in the HCT116 p53+/+ and p53-/- lines to determine what was occurring prior to 24 hours of treatment (Figure 4.10). The DNA damage seen by γ-H2AX began to increase around 6 to 12 hours of treatment and peaked at 24 hours. The p53 levels showed a similar pattern of induction to that of the γ-H2AX levels.

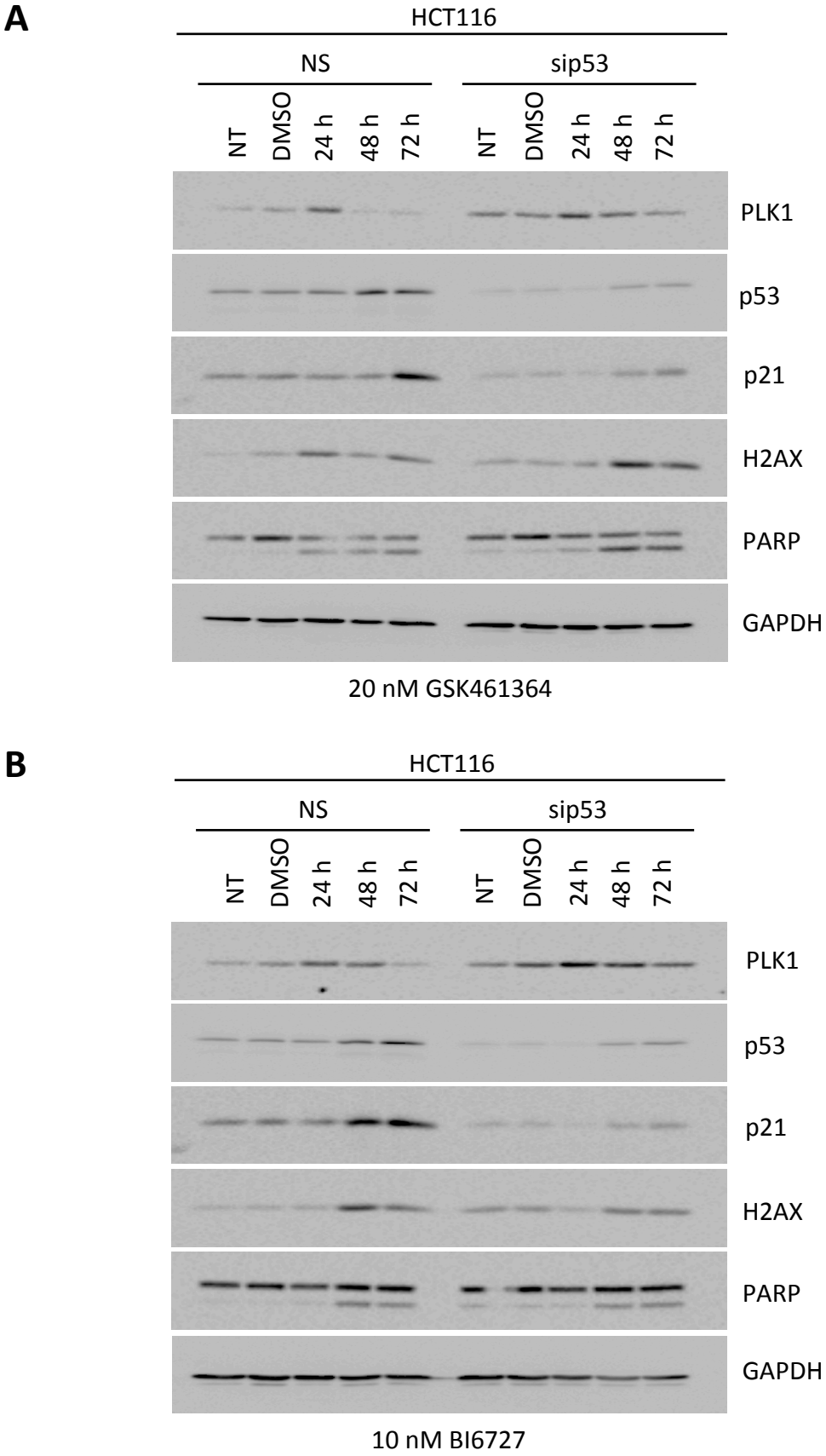


Figure 4.7. HCT116 cells treated with PLK1 inhibitors in p53 silenced conditions. HCT116 cells were transfected with non-silencing siRNA or siRNA targeting p53. After 24 hours incubation, cells were subsequently treated with 20 nM GSK461364 (A) or 10 nM BI6727 (B) for 24, 48 or 72 hours. As a control cells were left untreated or treated with DMSO (vehicle control). Cell lysates were then subjected to western blotting using the antibodies indicated in the figure.

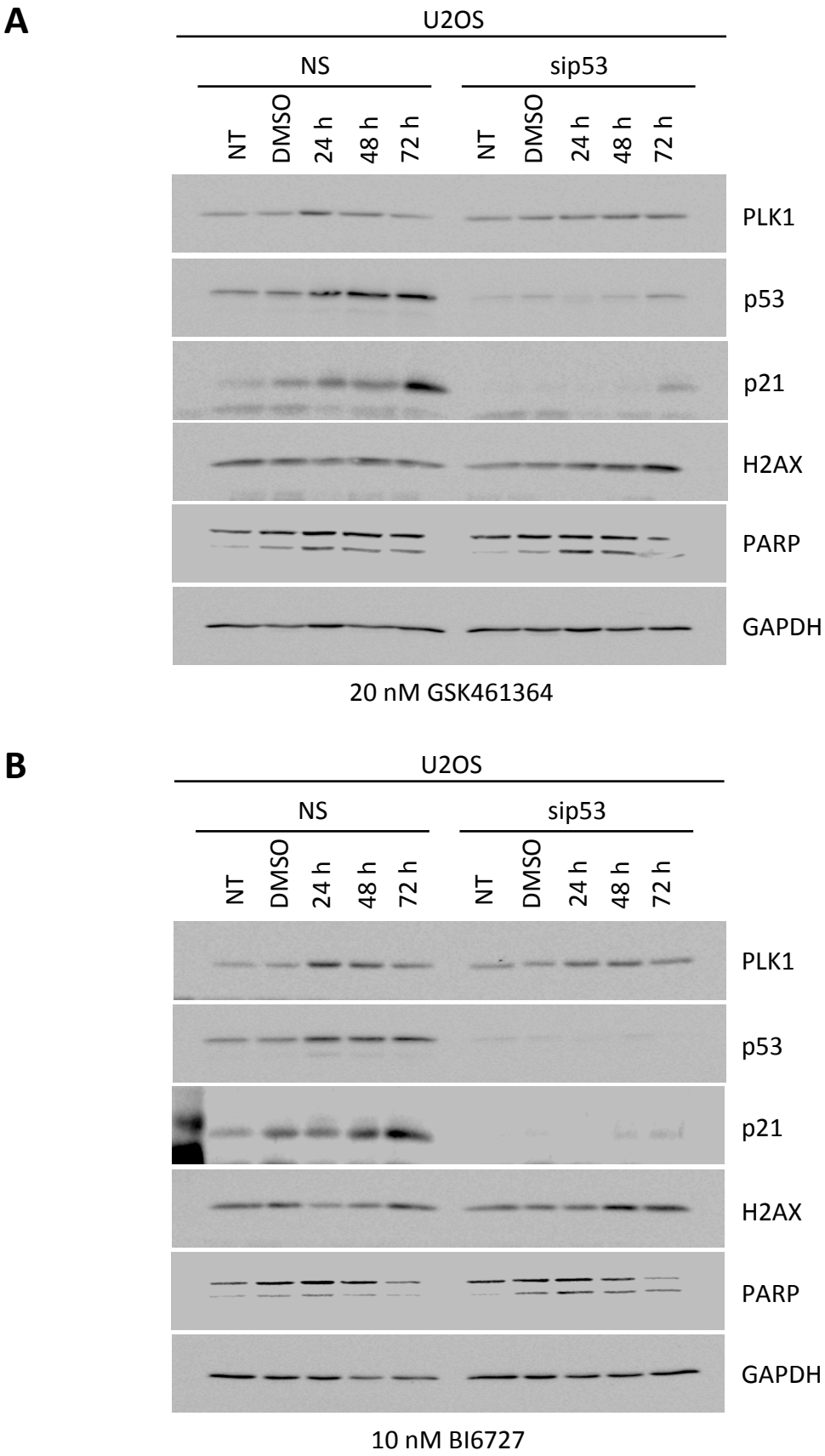


Figure 4.8. The effect of PLK1 inhibitors on U2OS cells with silenced p53. U2OS cells were transfected with non-silencing siRNA or siRNA targeting p53. After 24 hours incubation, cells were subsequently treated with 20 nM GSK461364 (A) or 10 nM BI6727 (B) for 24, 48 or 72 hours. As a control cells were left untreated or treated with DMSO (vehicle control). Cell lysates were then subjected to western blotting using the antibodies indicated in the figure.

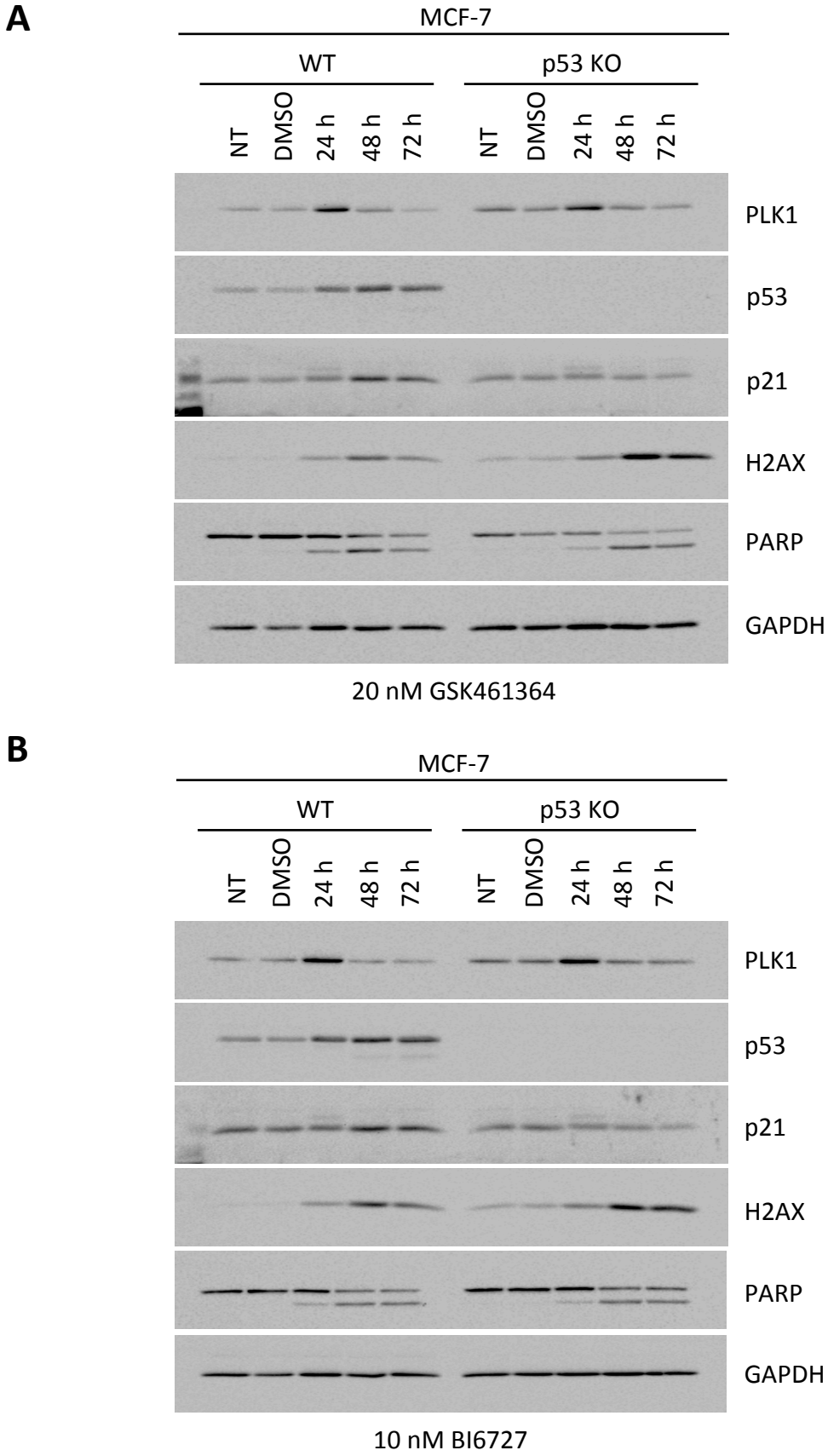


Figure 4.9. The effects of PLK1 inhibitors on wild type and p53 knockout MCF-7 cells. Parental MCF-7 cells and MCF-7 cells that have had p53 knocked out by use of CRISPR technology were untreated, treated with DMSO or treated with 20 nM GSK461364 (A) or 10 nM BI6727 (B) for 24, 48 or 72 hours. Cells were subsequently harvested and western blotting was used to assess protein levels using the antibodies indicated in the figure.

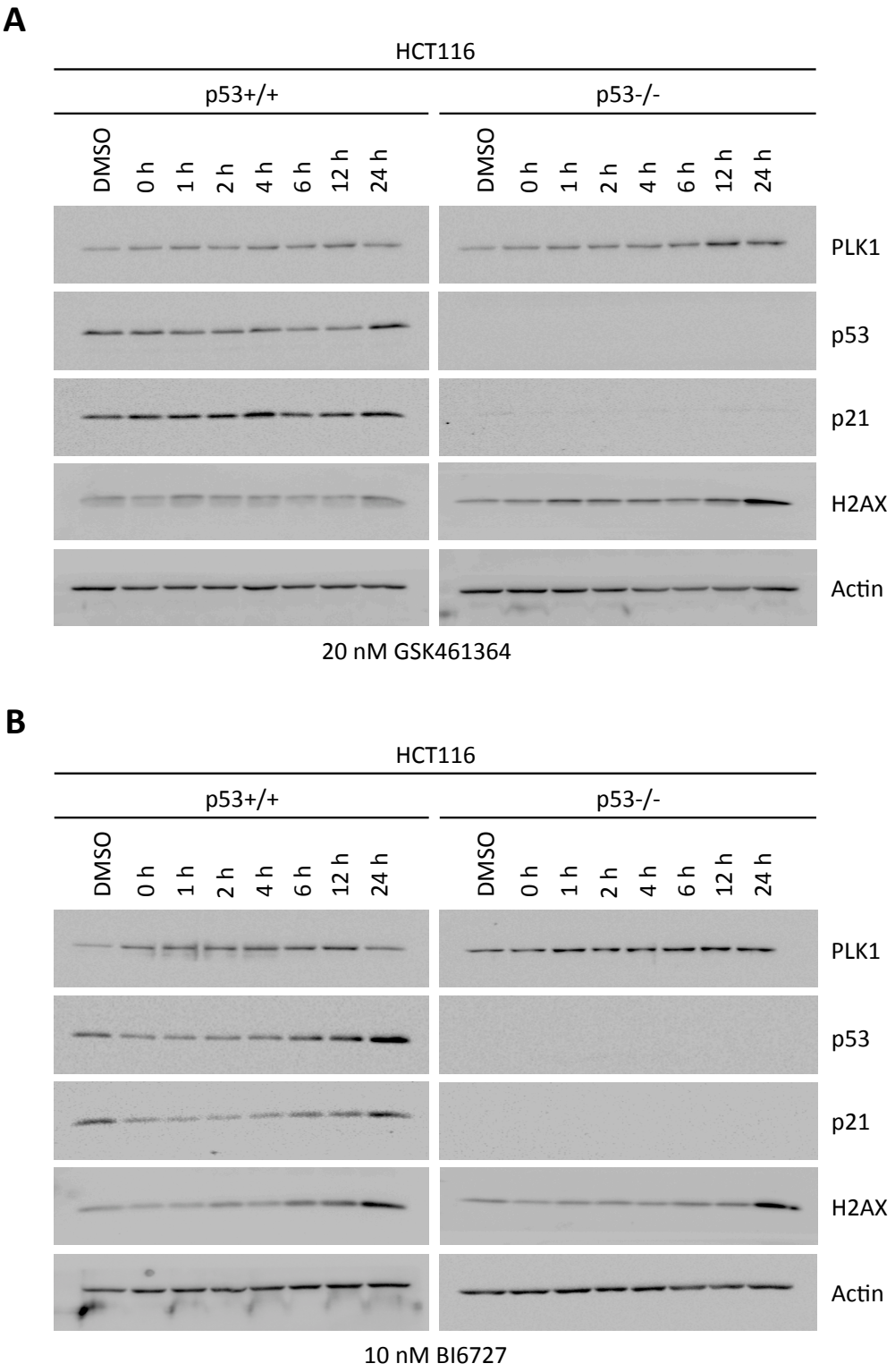


Figure 4.10. Inhibition of PLK1 in HCT116 cells results in induction of p53. HCT116 p53+/+ and p53-/- cells were treated with DMSO or treated for 0, 1, 2, 4, 6, 12 or 24 hours with 20 nM GSK461364 (A) or 10 nM BI6727 (B). Western blotting was then used with the antibodies indicated in the figure.

4.3.3. The DNA Damage response leads to activation of p53

PLK1 inhibitors are targeted protein kinase inhibitors and not standard genotoxic chemotherapeutic agents. Therefore the reason for observing a p53 response upon treatment with PLK1 inhibitors was uncertain. Numerous mechanisms can result in p53 activation. However, due to the observation that γ-H2AX increases upon treatment with PLK1 inhibitors, activation of p53 by the DNA damage response pathway seemed the most possible mode of p53 induction. Upon DNA damage occurring, the protein kinases ATM and ATR phosphorylate p53 at serine 15 to activate p53. Therefore, to investigate if the DNA damage response was responsible for activating p53 upon treatment with PLK1 inhibitors, inhibitors of ATM and ATR (KU-55933 and VE-821 respectively) were used. As a positive control, etoposide, a topoisomerase inhibitor that results in DNA damage and activation of p53 was used. As can be seen in Figure 4.11 treatment with GSK461364 or etoposide resulted in increased levels of p53 and phosphorylated p53 (serine 15) compared to the DMSO control. However, one hour pretreatment with KU-55933 and VE-821, followed by 24 hours of treatment with GSK461364 or etoposide reduced the total p53 levels and the levels of serine 15 phosphorylation. This strongly suggested that p53 was being activated by the DNA damage response pathways upon inhibition of PLK1.

To then determine if the DNA damage response was required for the G1 subset of cells observed by flow cytometry in wild type p53 cell lines, the ATM and ATR inhibitors were used, again with one hour of pretreatment, followed by 24 hours of treatment with GSK461364 or BI6727. Flow cytometry was then used to analyse the cell cycle distribution. Figure 4.12 shows that inhibition of the DNA damage response reduces the G1 peak, to a similar level as seen in the p53-/- line. (Additionally, the >4N peak was reduced with inhibition of ATM and ATR, although more cell death was seen

suggesting that due to lack of repair the cells die rather than arrest). Overall these findings suggest that activation of p53 through the DNA damage response is required for the accumulation of cells in G1 upon treatment with PLK1 inhibitors.

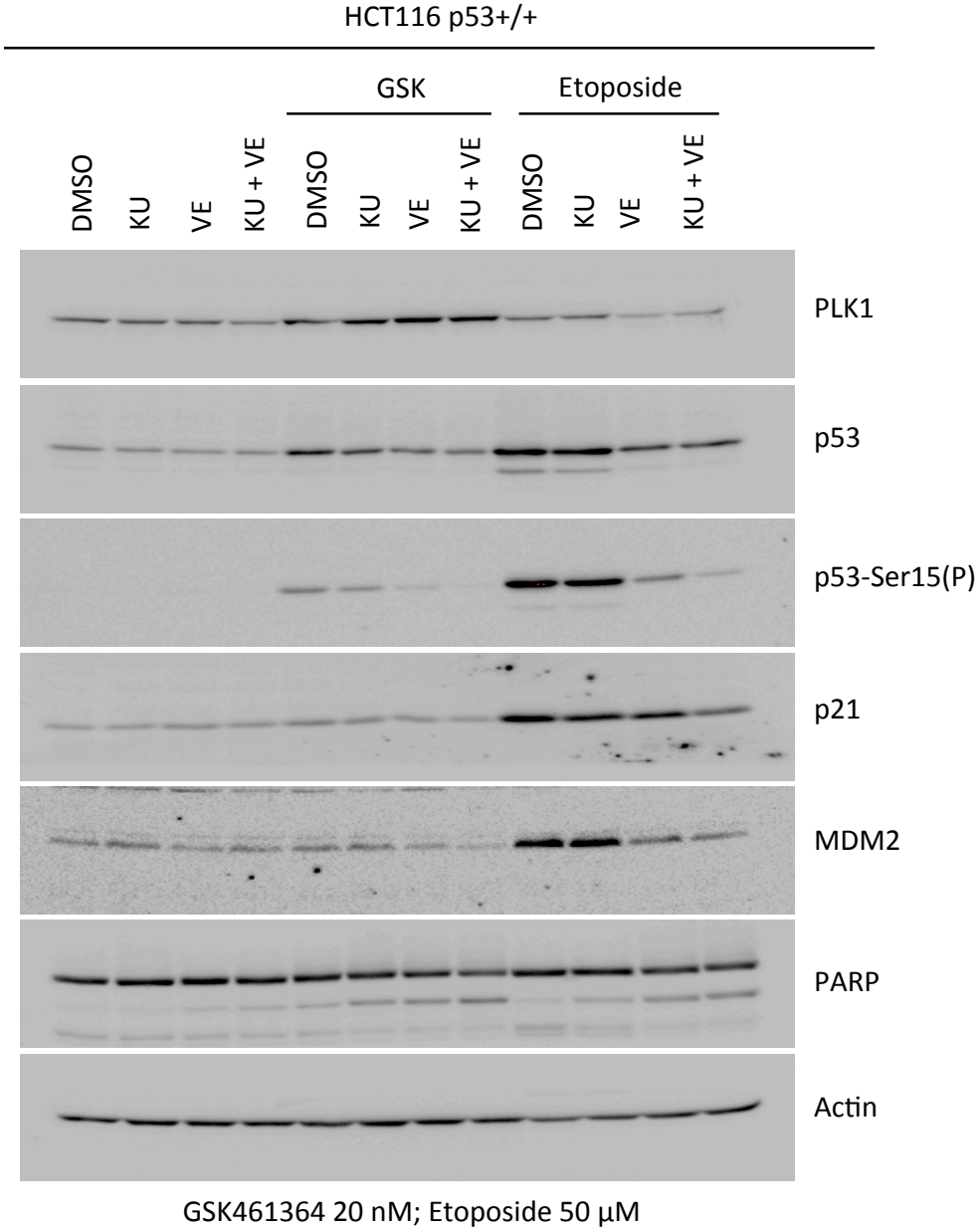
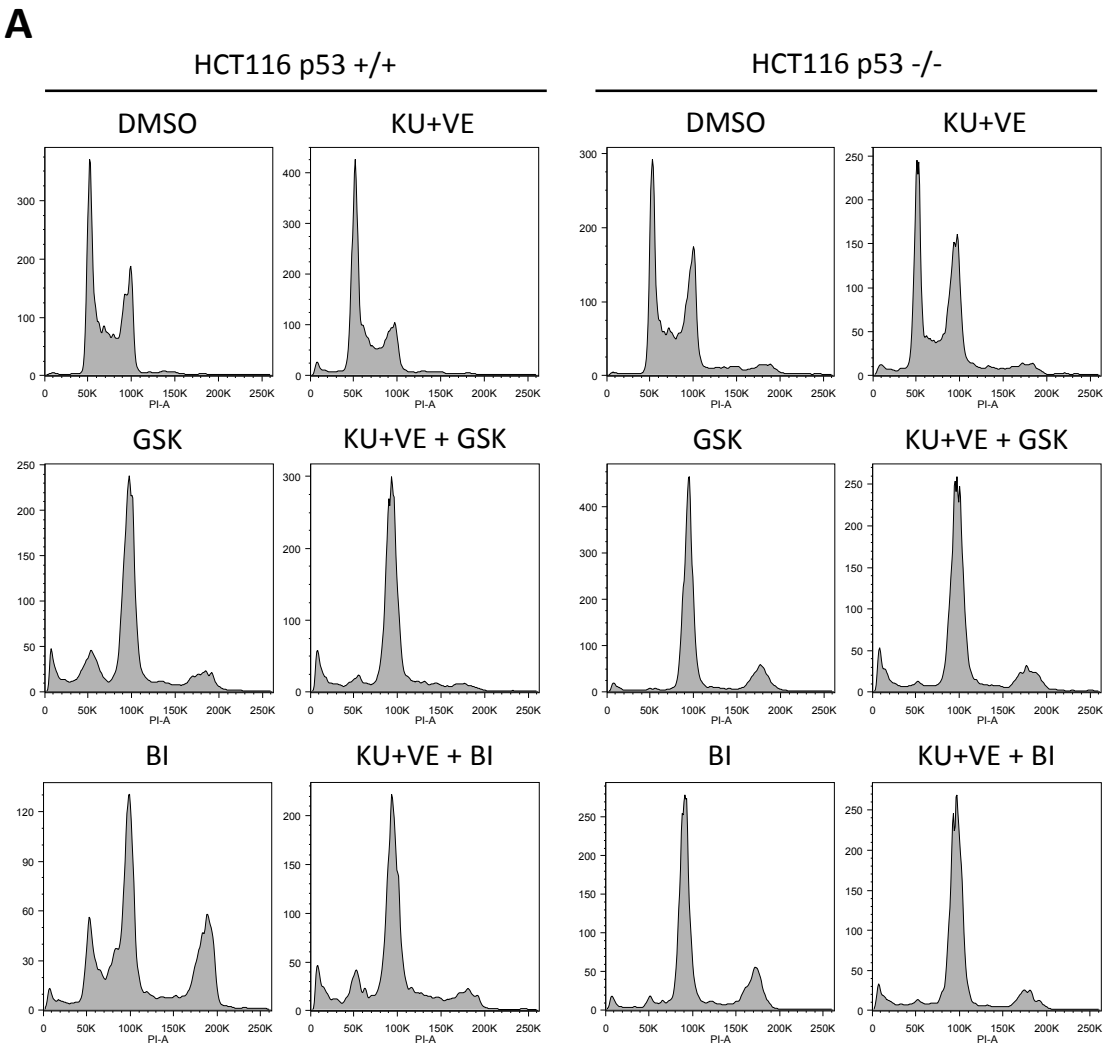
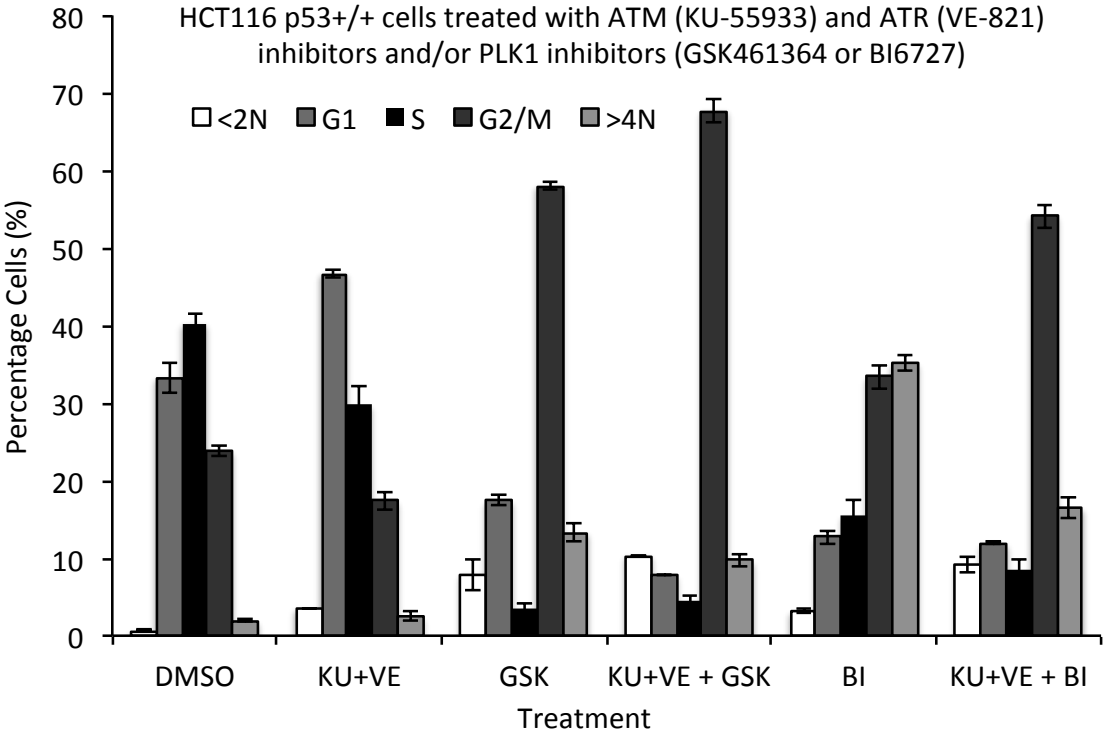


Figure 4.11. The DNA damage response pathway leads to induction of p53. HCT116 cells were pretreated for one hour with 10 µM ATM inhibitor, KU-55933, and/or ATR inhibitor, VE-821. Cells were subsequently treated for a further 24 hours with 20 nM GSK461364 or 50 µM etoposide before harvesting. Western blotting was used with the antibodies indicated in the figure.


A
HCT116 p53 +/+
HCT116 p53 -/-
DMSO
KU+VE
DMSO
KU+VE
GSK
KU+VE + GSK
GSK
KU+VE + GSK
BI
KU+VE + BI
BI
KU+VE + BI
PI-A


B
HCT116 p53+/+ cells treated with ATM (KU-55933) and ATR (VE-821) inhibitors and/or PLK1 inhibitors (GSK461364 or BI6727)
<2N
G1
S
G2/M
>4N
Percentage Cells (%)
DMSO
KU+VE
GSK
KU+VE + GSK
BI
KU+VE + BI
Treatment

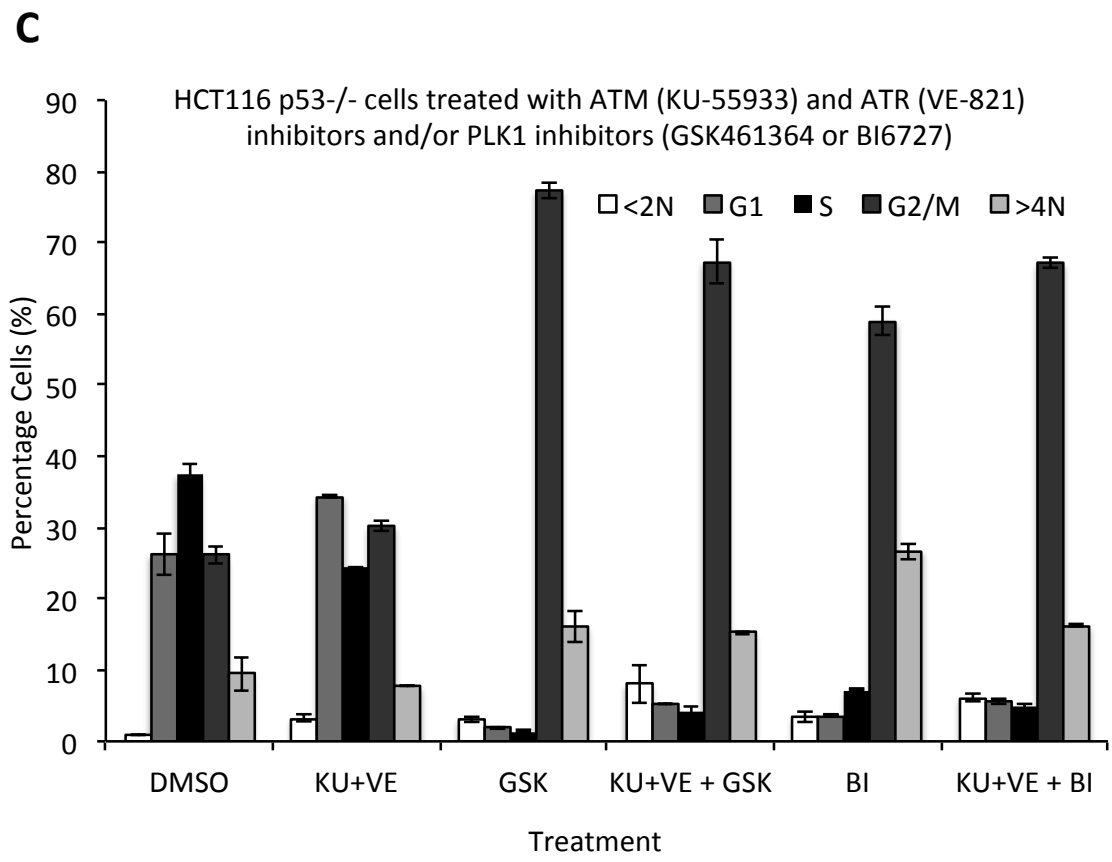


Figure 4.12. The DNA damage response pathway is required for p53 dependent cell cycle effects. HCT116 p53+/+ and p53-/- cells were treated with DMSO or 10 μM KU-55933 and VE-821 for one hour, before addition of DMSO, 20 nM GSK461364 or 10 nM BI6727 for a further 24 hours. Cells were subsequently harvested and stained for flow cytometry analysis. The resulting histograms are shown in (A) and quantification of the percentage of cells in each phase of the cell cycle is shown for HCT116 p53+/+ in (B) and HCT116 p53-/- in (C).

4.3.4. Phosphorylation of p53 at serine 15 is involved in the presence of the p53 dependent G1 subset of cells

Serine 15 has been shown to be the activating phosphorylation site of p53 upon DNA damage and is phosphorylated by ATM and ATR (Loughery *et al.*, 2014). Substitution of serine to alanine is a useful tool for blocking phosphorylation. Using the IPTG inducible Lacswitch II system, H1299 (a p53 null lung cancer cell line) were stably transfected to express either wild type p53 or p53 with a serine 15 to alanine substitution upon addition of IPTG. These cells could then be used to determine if serine 15 was essential for the previous p53 dependent results that had been observed. This would allow confirmation of the role of the DNA damage signalling pathways in activating p53 following inhibition of PLK1. Figure 4.13 shows that in the absence of IPTG p53 is not detected at a high level in either the wild type or S15A cells. However upon addition of IPTG there is a dose dependent increase in p53 levels. HCT116 cells have been included as a control for comparison of endogenous levels of p53, to ensure the system is not giving rise to overexpressed levels of p53. Loss of serine 15 phosphorylation impairs the ability of p53 to induce downstream target genes such as p21, as seen in Figure 4.13. Next, flow cytometry, using the same experimental set up as previously shown, was used. As can be seen in Figure 4.14 under DMSO conditions both the H1299 S15 and S15A cell lines had similar profiles upon addition of IPTG. As a control the H1299 p53 null parental cell line was also tested. Figure 4.14 shows that the parental H1299 cells have an increase in the proportion of cells in S phase and decrease in the proportion of cells in G1 under DMSO conditions, compared to the inducible H1299 lines. Upon treatment with PLK1 inhibitors a similar result was again observed, with the wild type p53 (S15WT) cells showing an increased proportion of cells in G1 in comparison to the S15A cells or H1299 parental cells. This suggests that S15 is playing a role in the outcome of inhibition of PLK1.

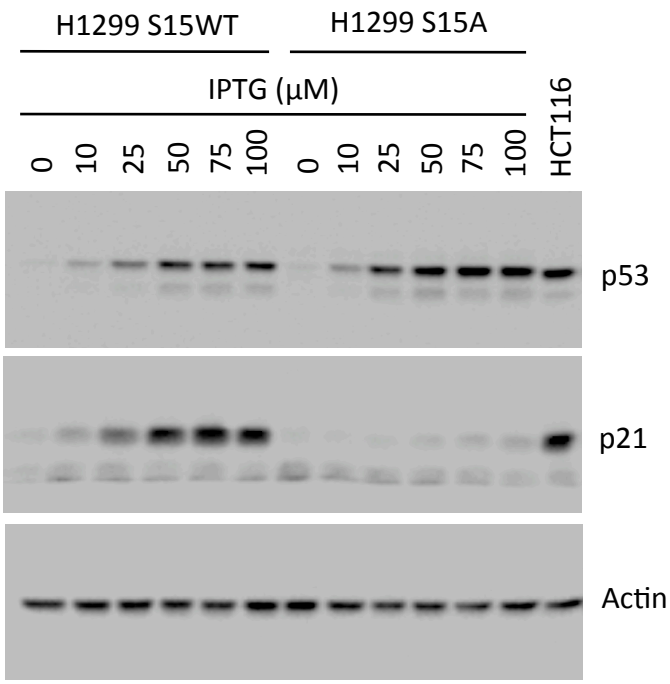


Figure 4.13. Induction of p53 in H1299 p53 inducible lines. IPTG inducible H1299 cells expressing wild type p53 or p53 with serine 15 substituted to alanine were treated with 0, 10, 25, 50, 75 or 100 μM IPTG for 16 hours. Cells were harvested and western blotting was used to analyse the levels of p53, p21 and actin protein levels. HCT116 cells (wild type p53) were used as a reference for endogenous p53 levels.

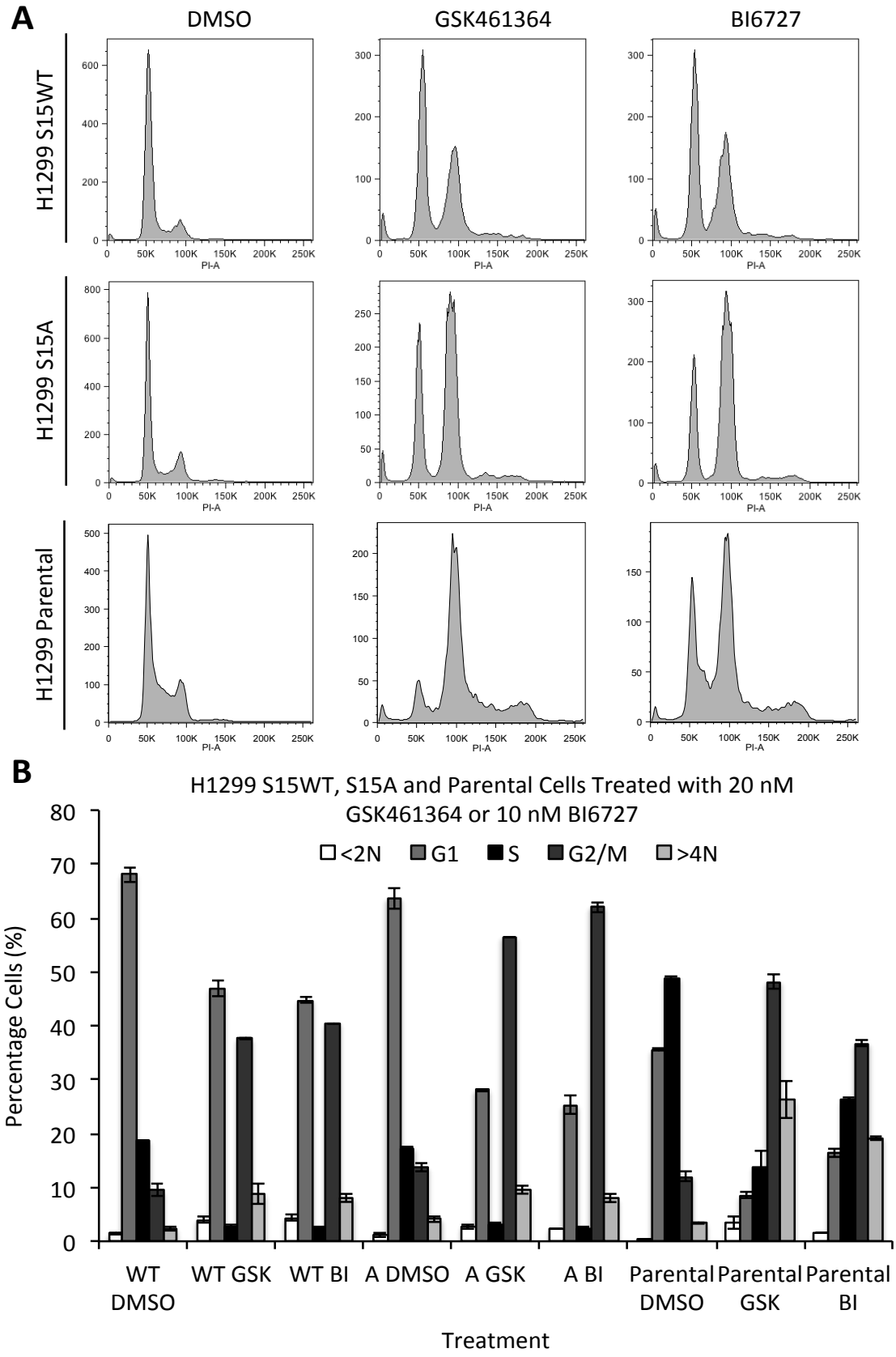


Figure 4.14. p53 serine 15 is involved in the accumulation of cells in G1. Parental H1299 cells or inducible H1299 cells expressing wild type p53 or p53 S15A were treated with 100 μM IPTG for 16 hours. Cells were then treated with DMSO, 20 nM GSK461364 or 10 nM BI6727 for a further 24 hours, with 100 μM IPTG being maintained throughout. Cells were harvested and flow cytometry was used to assess the cell cycle distribution. Resulting histograms (A) and quantification of cells in each phase of the cell cycle (B) are shown.

4.3.5. PLK1 Inhibition leads to DNA Damage

As shown, the two inhibitors of PLK1 led to DNA damage as observed by induction of γ-H2AX. However, small molecule kinase inhibitors are notoriously promiscuous, and often have a lack of specificity and therefore off target effects can occur. To inhibit PLK1 by a different method, to see if the DNA damage was occurring as a specific result of inhibiting PLK1 or an off target effect of the inhibitors, siRNA targeting PLK1 was utilised. A mock treatment, using the transfection reagents with no siRNA, non-silencing siRNA and siPLK1 were used. In addition, as a comparison, treatments with GSK461634 and BI6727 were also used. Figure 4.15 shows that silencing PLK1 with siRNA results in γ-H2AX induction. When compared to treatment with either PLK1 inhibitor, it can be seen that the levels of γ-H2AX are fairly similar, as are the levels of cleaved PARP. This suggested that the DNA damage occurring was a direct result of inhibiting PLK1 and not an off target effect. Although, the induction of p53 is much less with silencing of PLK1 compared to treatment with PLK1 inhibitors. As an additional control, the samples that were transfected with PLK1 siRNA, were treated with the PLK1 inhibitors in combination. This would allow determination of any additive effects, which would again suggest off target effects were occurring. If the DNA damage resulting from knocking down PLK1 was a result of a different source from the DNA damage caused by the inhibitors, then double the amount of DNA damage would have been expected. However, as the figure shows, the γ-H2AX levels with combination of PLK1 siRNA and PLK1 inhibitor are consistent with knocking down PLK1 or treating with PLK1 inhibitor alone. This strongly implies the PLK1 inhibitors are specific, and inhibiting PLK1 results in DNA damage.

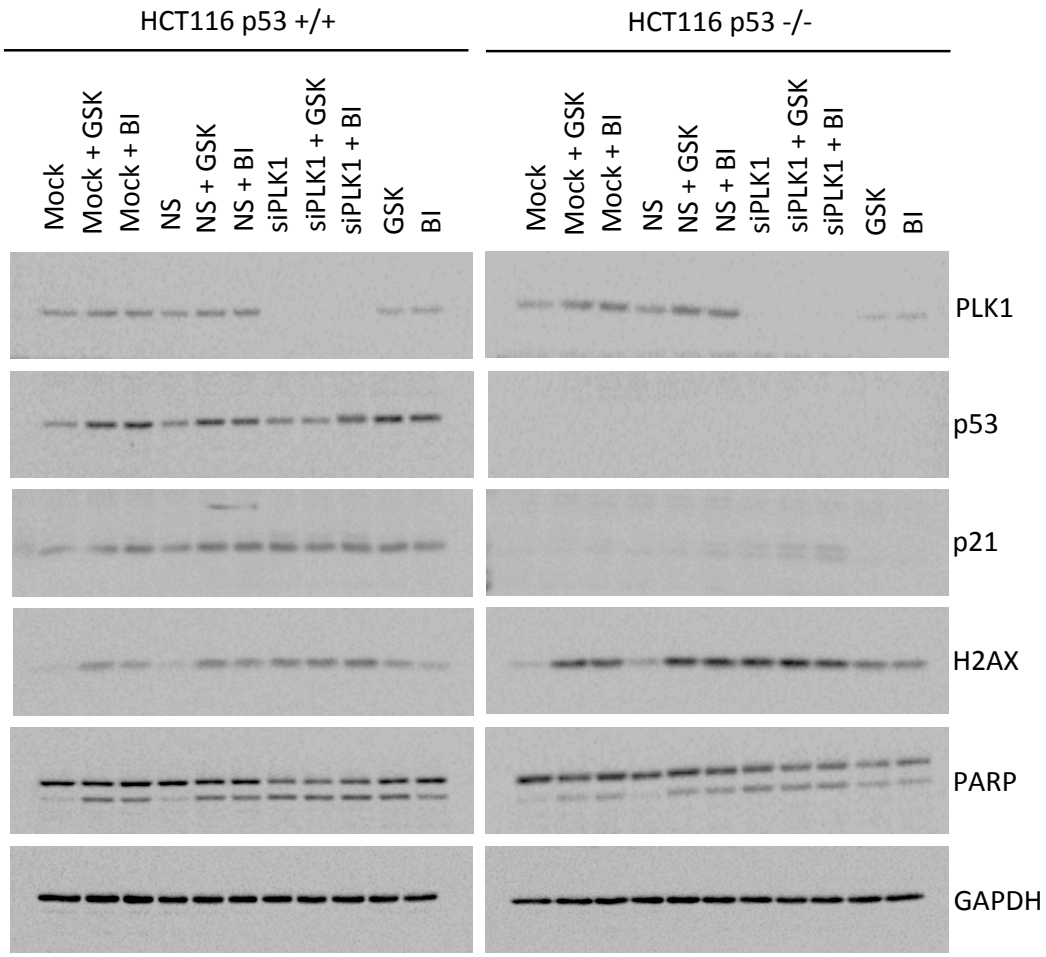


Figure 4.15. Inhibition of PLK1 leads to DNA damage. HCT116 p53+/+ and p53-/- cells were transfected with a mock transfection (transfection reagent alone), non-silencing siRNA or siRNA targeting PLK1. After 24 hours cells were treated with DMSO, 20 nM GSK461364 or 10 nM BI6727 for a further 24 hours. Cells were then harvested and western blotting was used with the antibodies indicated in the figure.

4.3.6. The DNA damage is not caspase dependent

Some reports show that DNA damage occurring as a result of prolonged mitosis is caspase dependent and p53 recognises and responds to this damage in the subsequent G1 after mitotic exit (Hain *et al.*, 2016). Z-VAD-FMK, a pan caspase inhibitor, blocks the initial stages of activation of apoptosis. This prevents cells undergoing apoptosis as a method of cell death. To address if the DNA damage observed is caspase dependent and a result of initiation of apoptosis, cells were pre-treated for one hour with Z-VAD-FMK. GSK461364 or BI6727 were then added to the cells for a further 24 or 48 hours. As can be seen in Figure 4.16 Z-VAD-FMK successfully blocks apoptosis as seen from the reduction of cleaved PARP. Whilst apoptosis is inhibited, the DNA damage observed is very similar in level in the presence or absence of Z-VAD-FMK. This shows that the DNA damage occurring with treatment of PLK1 inhibitors is not likely to be caspase dependent and a consequence of apoptosis.

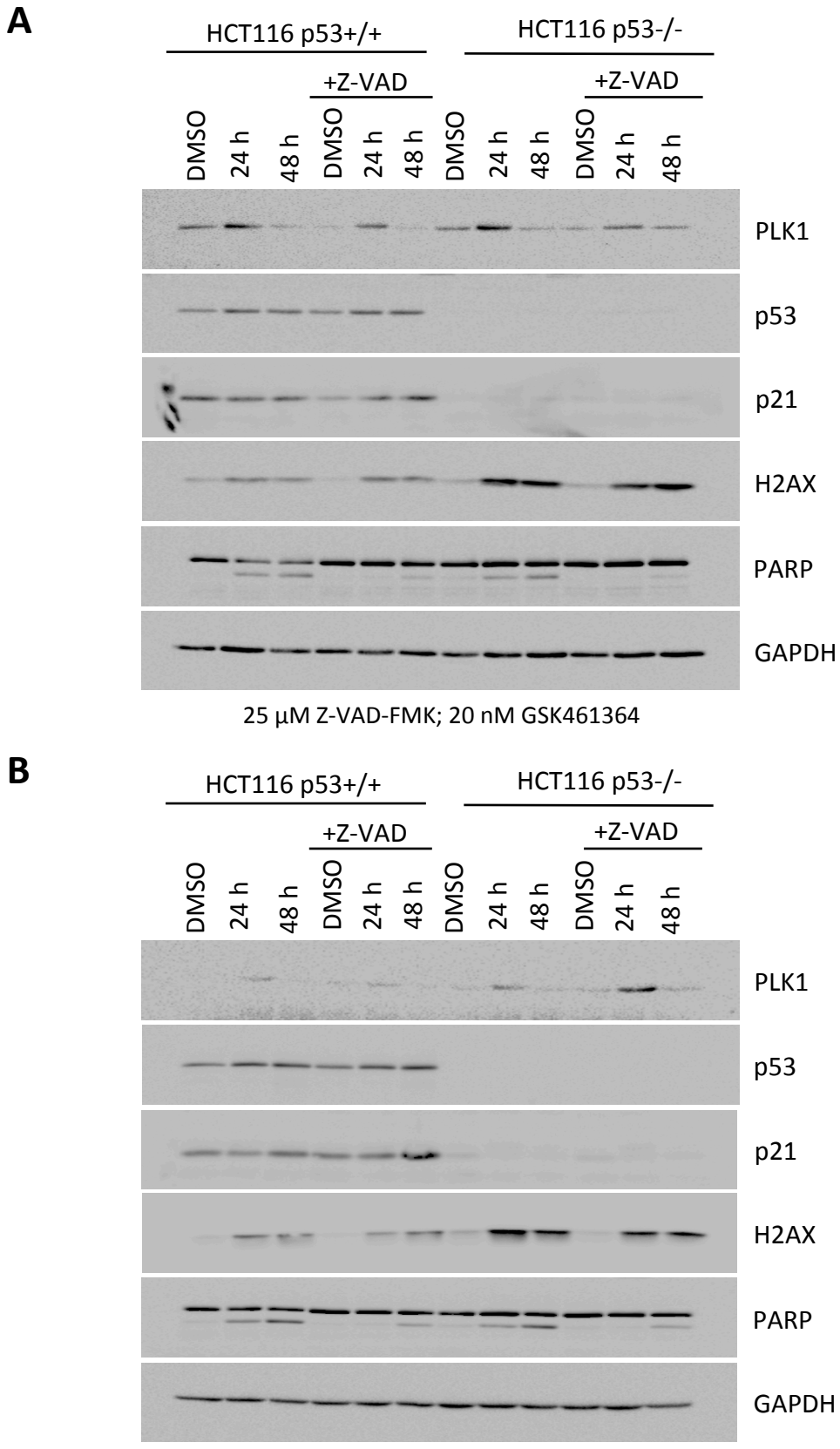


Figure 4.16. The DNA damage is not caspase dependent. HCT116 p53+/+ and p53-/- cells were pretreated for one hour with 25 µM Z-VAD-FMK or DMSO (vehicle control) before addition of (A) 20 nM GSK461364 or (B) 10 nM BI6727 for 24 or 48 hours. Cells were then harvested at the indicated time points and western blotting was used to assess protein levels using the antibodies shown in the figure.

4.4. Discussion

This chapter has shown that when PLK1 inhibitors are applied, p53 is induced. Further experiments have shown that this induction of p53 occurs through the DNA damage response pathways, as inhibition of ATM and ATR (two DNA damage response protein kinases) alleviates the activation of p53. The DNA damage resulting from PLK1 inhibition shown here is in agreement with previous studies that have also observed DNA damage occurring as a result of PLK1 inhibition (Driscoll *et al.*, 2014). Additionally, the DNA damage was shown to be specific to inhibition of PLK1 and not an off target effect of the ATP competitive small molecule inhibitors tested, as use of siRNA targeting PLK1 also resulted in DNA damage. This DNA damage could therefore be the activating stimulus for p53, and may be required for the p53 mediated protective effect. A DNA damage response has previously been shown to occur upon prolonged mitotic delay. However, this was suggested to be caspase dependent (Hain *et al.*, 2016). Use of a pan-caspase inhibitor showed that the DNA damage occurring in response to PLK1 inhibitors did not appear to be caspase dependent, therefore suggesting that this was not the cause of DNA damage in this case. Further investigations should therefore be carried out to determine the cause of the DNA damage observed upon inhibition of PLK1.

The p53-dependent differences observed in cell cycle distribution upon treatment with PLK1 inhibitors highlight a plausible source of protection through the identification of a p53-dependent G1 subset of cells. It has been shown that cells which do not successfully complete mitosis and go on to re-enter the cell cycle with an abnormal chromosome complement will be arrested by p53, and most likely undergo apoptosis (Hayashi and Karlseder, 2013). This therefore rules out cells with a complement of DNA greater than 2N as being cells likely to recover and survive PLK1 inhibition in a

p53-dependent manner. This leaves cells arrested in G2 or mitosis and cells accumulating in G1 as the only plausible surviving cells. However, cells are also arrested in G2/mitosis in the absence of p53, which further implicates the G1 cells as being a more probable source of p53 dependent protection. It should be noted that functional isoforms of p53 exist, and some of these isoforms are present in the HCT116 lines. The HCT116 p53-/- line, whilst lacking full-length p53 expression, does express shorter p53 isoforms (Murray-Zmijewski, Lane and Bourdon, 2006). The effects observed could potentially be due to the activity of the isoforms. However, the siRNA used to silence p53 targets exon 7 of p53. By targeting this exon all p53 isoforms are silenced, therefore suggesting that the effects observed are due to full-length p53. The G1 subset of cells are reliant on activation of p53, as inhibiting ATM and ATR or substituting the critical residue on p53, serine 15, to alanine results in a cell cycle distribution similar to that of cells lacking p53. Although activation of p53 is required by ATM and ATR to exert p53 dependent effects in regards to PLK1 inhibition, it is not known whether the DNA damage induced by PLK1 inhibition is a critical stimulus for this response or whether basal levels of p53 activity or some other stimulus are sufficient to exert such an effect. Further work could attempt to address this question, and could also determine if serine 15 is the only critical residue, or if other sites of post-translational modifications are equally as important.

Chapter 5 : p53 plays a novel role in centrosome separation during early mitosis

5.1. Background

One of the results of inhibition of PLK1 is a p53 response, ultimately leading to decreased sensitivity to PLK1 inhibitors. However, the mechanism by which p53 elicits this protective effect is unclear. The data shown above, suggests that a subset of p53 competent cells can accumulate in G1 upon treatment with PLK1 inhibitors. However, the mechanism behind this is unknown. PLK1 has been shown to have roles in DNA replication in S phase (H. Yim and Erikson, 2009; Song *et al.*, 2011; Shen *et al.*, 2013), there is therefore potential for a p53 dependent effect in the early parts of the cell cycle. This could be the reason for G1 cells to accumulate in a p53-dependent fashion. Alternatively, these cells may be able to complete mitosis even in the presence of PLK1 inhibitors. It has been widely accepted that during mitosis, transcription is repressed due to the highly condensed chromatin, making promoters inaccessible, and the displacement of specific RNA polymerase II factors from the chromosomes (Gottesfeld and Forbes, 1997). This would suggest that as a transcription factor, p53 would not be able to exhibit any effects during mitosis. Although one study suggested that some transcriptional activity can remain during mitosis, as Cyclin B1 was shown to be transcribed during mitosis in Hela cells (Sciortino *et al.*, 2001). There are therefore several possible ways in which p53 may allow the accumulation of cells in G1 upon treatment with PLK1 inhibitors. As this is the most plausible means by which p53 provides a protective effect to some cancer cells upon application of PLK1 inhibitors, the mechanism by which this occurs is of great interest.

Time-lapse microscopy has become a highly useful tool for investigating mitosis and other aspects of cell movements. Generation of movies allows analysis of individual cells, and can allow determination of timings of the cell cycle, with particular focus on mitotic duration. As PLK1 inhibitors result in a mitotic arrest, it is therefore beneficial

to be able to interrogate mitosis in such a way. Time-lapse microscopy has been effectively utilised throughout this chapter, along with immunofluorescence, which still provides an excellent method of determining the localisation of specific proteins within a cell.

5.2. Aims

The aim of this chapter is to attempt to determine the mechanism(s) that results in the p53-dependent accumulation of a subset of cells in G1 upon treatment with PLK1 inhibitors, as this could potentially underpin the protective effect that p53 offers some cancer cells as observed previously.

5.3. Results

5.3.1. Cells accumulating in G1 in a p53 dependent manner complete mitosis

While there are few reports on the role of PLK1 outwith mitosis, some publications implicate PLK1 as having a role in S phase (Song *et al.*, 2011; Song, Liu and Liu, 2012; Mandal and Strebhardt, 2013). Therefore, we wondered if the DNA damage was actually occurring earlier in the cell cycle upon treatment with PLK1 inhibitors and this resulted in the p53 dependent G1 cells observed. Cells were synchronised in order to investigate whether the DNA damage occurs earlier in the cell cycle, and thus results in cells arresting at G1/S prior to completing mitosis. Serum starvation was used as a method of synchronising the cells at entry to G1. The first step was to estimate how long it takes the cells to reach different phases of the cell cycle. Flow cytometry was used to assess this, with cells being serum starved for 24 hours, before re-addition of serum to allow entry into G1, and harvesting at different time points. Figure 5.1 shows that cells reach the G1/S boundary by around 6 hours, are mainly in S phase at 12 hours,

M phase at 16 hours and the full cycle is completed by 24 hours. However, the population begins to become asynchronous very quickly.

Using these times as a basis, cells were serum starved for 24 hours before re-addition of serum in the presence of DMSO or 20 nM GSK461634 for 0, 3, 12, 16 and 24 hours. Figure 5.2 shows that in the presence of DMSO, the HCT116 p53+/+ and p53-/- cells have very similar profiles at each of the time points tested. In the presence of GSK461364 the profiles are also very similar at 0, 3, 12 and 16 hours, however at 24 hours differences begin to emerge. The G1 peak in both the p53+/+ and p53-/- cells is almost absent at 16 hours, but at 24 hours it increases in size only in the p53+/+ cells. The G1 peak must therefore arise from cells that have successfully undergone cytokinesis in the presence of the PLK1 inhibitor, and not as a result of arresting prior to entry into S phase.

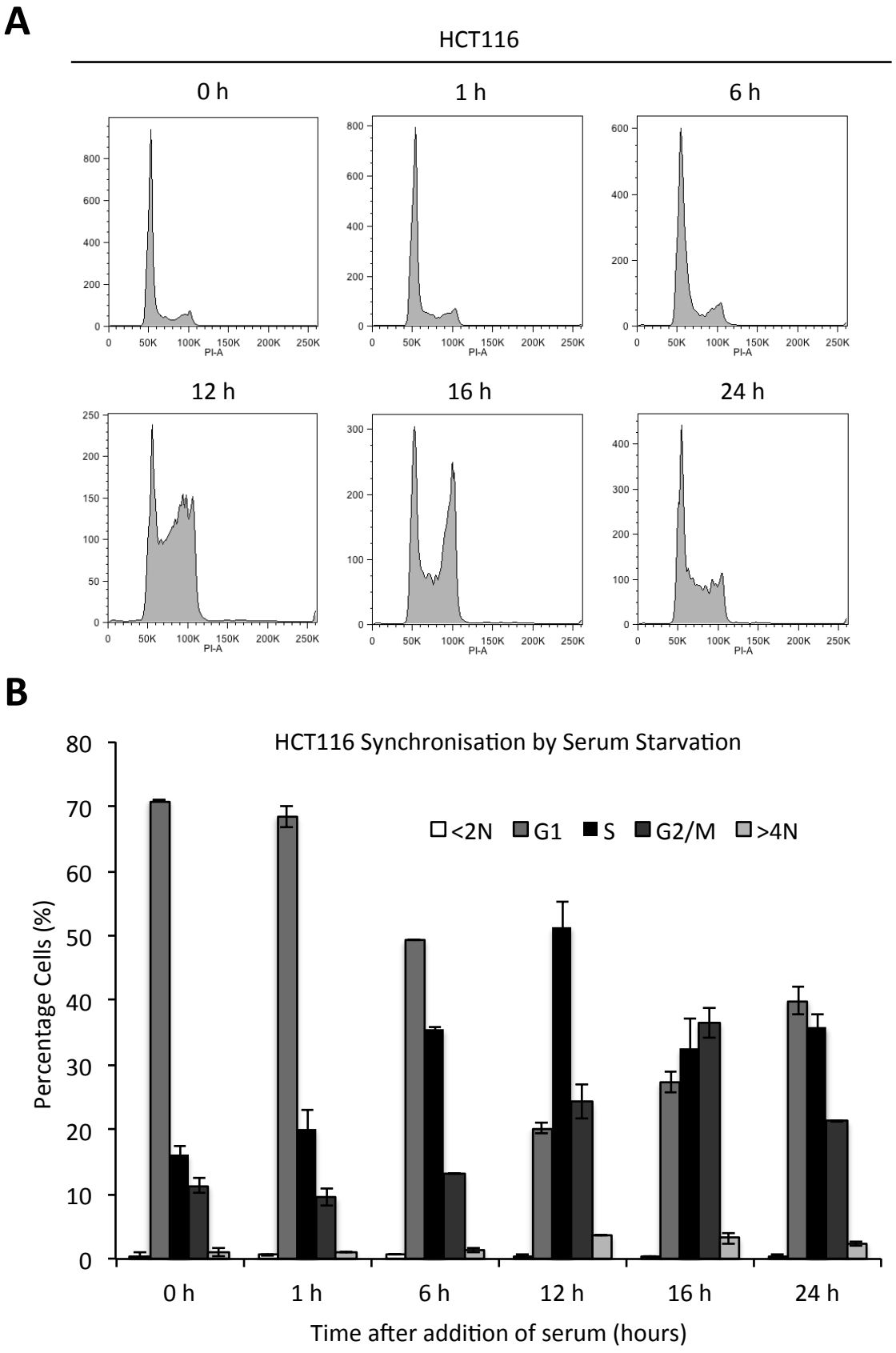
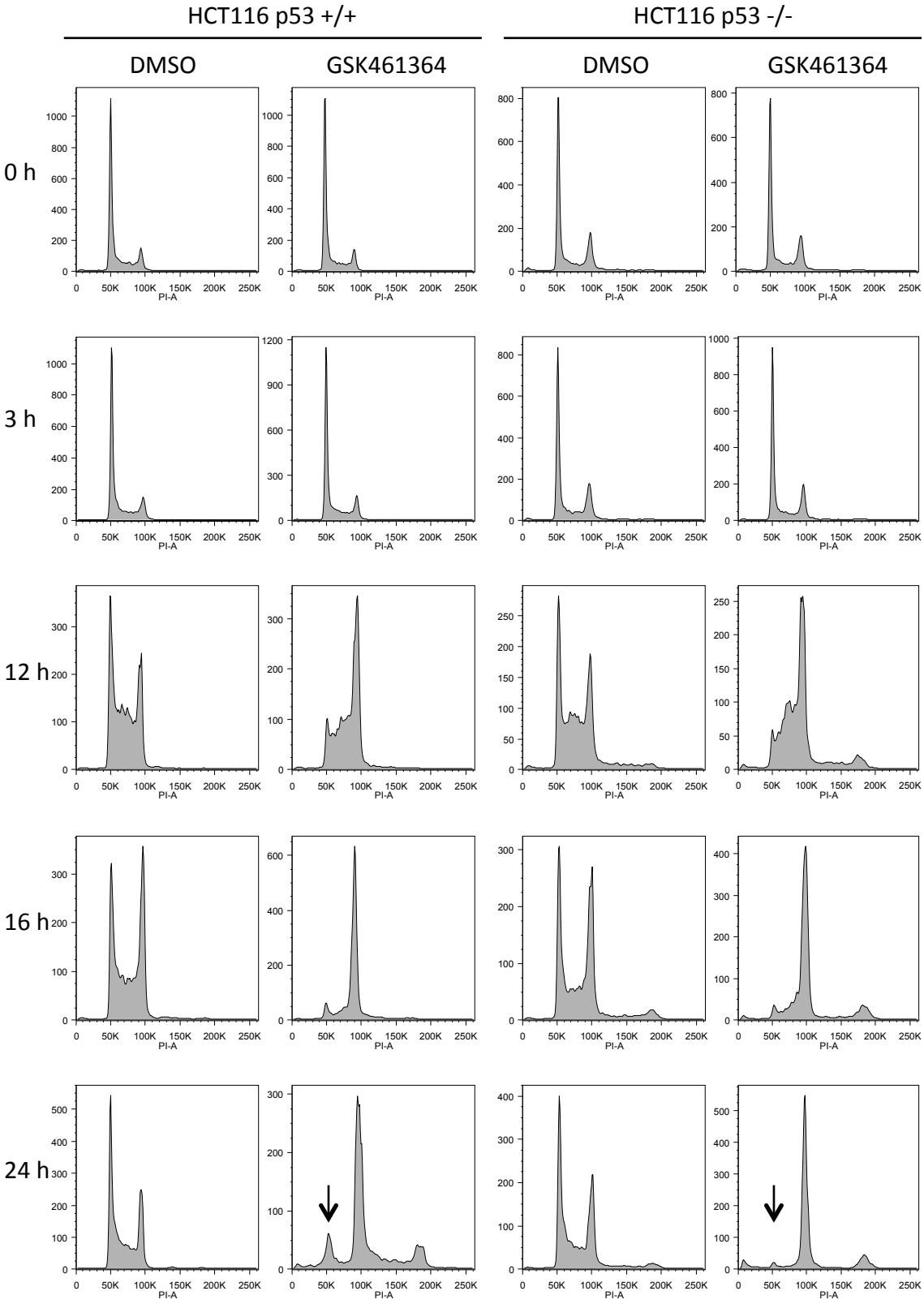


Figure 5.1. Synchronisation of HCT116 cells. HCT116 cells were serum starved for 24 hours. Serum was then reintroduced to the cells before harvesting at 0, 1, 6, 12, 16 or 24 hours after the addition of serum. Flow cytometry was then used to assess the cell cycle profiles. Resulting histograms are shown in (A) and the quantification of this data is represented in (B).

A


HCT116 p53 +/+
HCT116 p53 -/-
DMSO
GSK461364
DMSO
GSK461364
0 h
3 h
12 h
16 h
24 h
PI-A

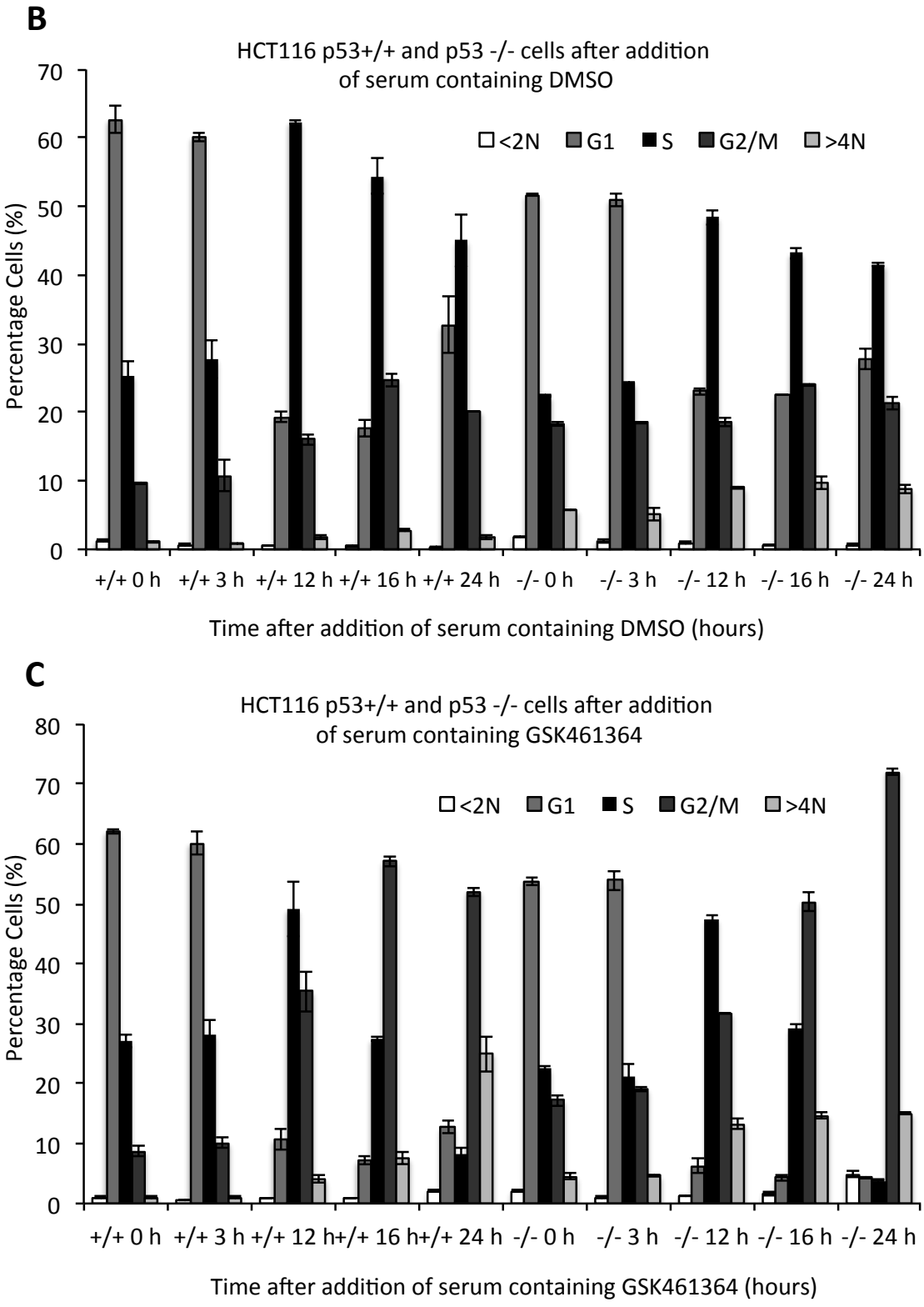


Figure 5.2. The G1 subset of cells arise from cells undergoing mitosis in the presence of GSK461364. HCT116 p53+/+ and p53-/- cells were serum starved for 24 hours. Serum was then added back to cells for one hour before the addition of DMSO or 20 nM GSK461364. Cells were subsequently harvested at 0, 3, 12, 16 or 24 hours. Flow cytometry was used to assess the cell cycle profiles. Resulting histograms are shown in (A) and the quantification of this data is represented for DMSO in (B) and GSK461364 in (C).

5.3.2. p53 reduces the duration of mitotic arrest upon inhibition of PLK1

As it was shown that the p53-dependent subset of cells appearing in G1 after treatment with PLK1 inhibitors were arising from completing mitosis in the presence of the drug, the next aim was to determine if the mitotic arrest was different in the absence or presence of p53. To do this, HCT116 p53+/+ and p53-/- cells were used. Cells were seeded in a 24-well plate format, and treated with different doses of GSK461364 or BI6727, or treated with DMSO as a vehicle control. Following treatment, the plate was mounted on the Zeiss Axiovert microscope. Positions were then set within each well to allow the progress of the cells to be followed in phase contrast. The cells were imaged every 4 minutes over 250 frames. This allowed the production of movies in which cells could be seen rounding up to indicate entry of mitosis through to the decision of cellular fate, either successful cytokinesis, apoptosis, mitotic slippage or mitotic arrest. To determine if differences existed between the two HCT116 lines, the duration between entry to mitosis and exit from mitosis (successful cytokinesis, apoptosis or mitotic slippage) was measured. For each condition 50 cells entering mitosis were followed and the resulting times taken to reach mitotic exit were plotted on graphs.

Figure 5.3 shows that under DMSO conditions, HCT116 p53+/+ and p53-/- cells have a short mitotic duration, with 100% of cells exiting mitosis in the time frame analysed. The two cell lines show little difference in their mitotic duration under normal, unstressed conditions. Next, the effect of increasing doses of GSK46164 was assessed. 5 nM, 10 nM and 20 nM concentrations of GSK461364 were tested. In the HCT116 p53+/+ line 5 nM of drug results in a slight increase in mitotic duration. 10 nM causes a further delay in mitotic exit, with 20 nM causing the most adverse effect on mitotic duration. Not only was there a delay in mitotic exit with increasing concentrations of GSK461364, there was also a decreased number of cells ever exiting mitosis in the time

frame followed. GSK461364 clearly results in a mitotic arrest with increased severity in a dose dependent fashion. Next, the HCT116 p53-/- cells were analysed in the same format. Again GSK461364 resulted in a mitotic arrest with increasing severity in a dose dependent manner. Although under DMSO conditions there is little difference between the mitotic durations, upon treatment with PLK1 inhibitor the p53+/+ cells show a shorter delay in mitosis compared to the p53-/- cells. This could suggest that the PLK1 inhibitor has less effect on wild type p53 cells, somehow allowing them to complete mitosis at a rate more similar to the normal homeostatic conditions. However, in the absence of p53 cells are delayed in mitosis for a much longer time, preventing cells returning to G1. This supports the observations of the flow cytometry data that show wild type p53 results in a G1 subset of cells that is not present in p53 deficient cells.

BI6727 was also used in the same assay, with a similar trend of results to that of treatment with GSK461364, as shown in Figure 5.4. This shows that the effects observed are not specific to one PLK1 inhibitor, but occur with two independently developed PLK1 inhibitors.

Although a very interesting observation, this could be a general consequence of inhibiting mitosis, rather than a specific effect of PLK1 inhibition. In order to check this, Taxol was used in the same experiment. Figure 5.5 shows that whilst increasing concentrations of Taxol results in an increased delay in mitotic exit, there is little difference in the duration of mitosis between the p53+/+ and p53-/- lines upon treatment with Taxol. This suggests that the effect observed is more specific to inhibition of PLK1 and not a general effect of inhibiting mitosis.

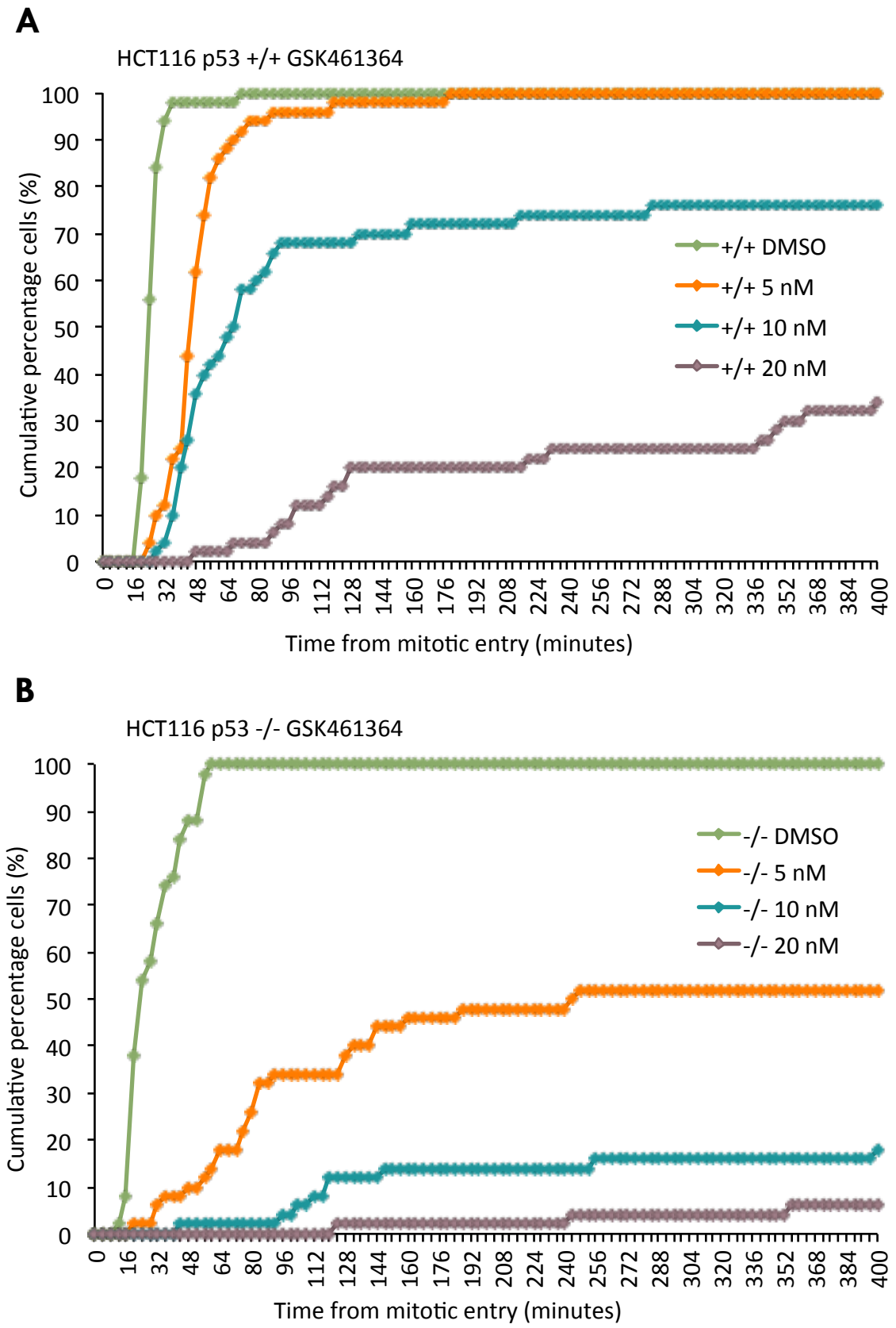


Figure 5.3. p53 reduces the duration of mitotic arrest upon treatment with GSK461364. HCT116 p53+/+ (A) and p53-/- cells (B) were treated with 5, 10 or 20 nM GSK461364 or DMSO as a vehicle control. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of three replicates.

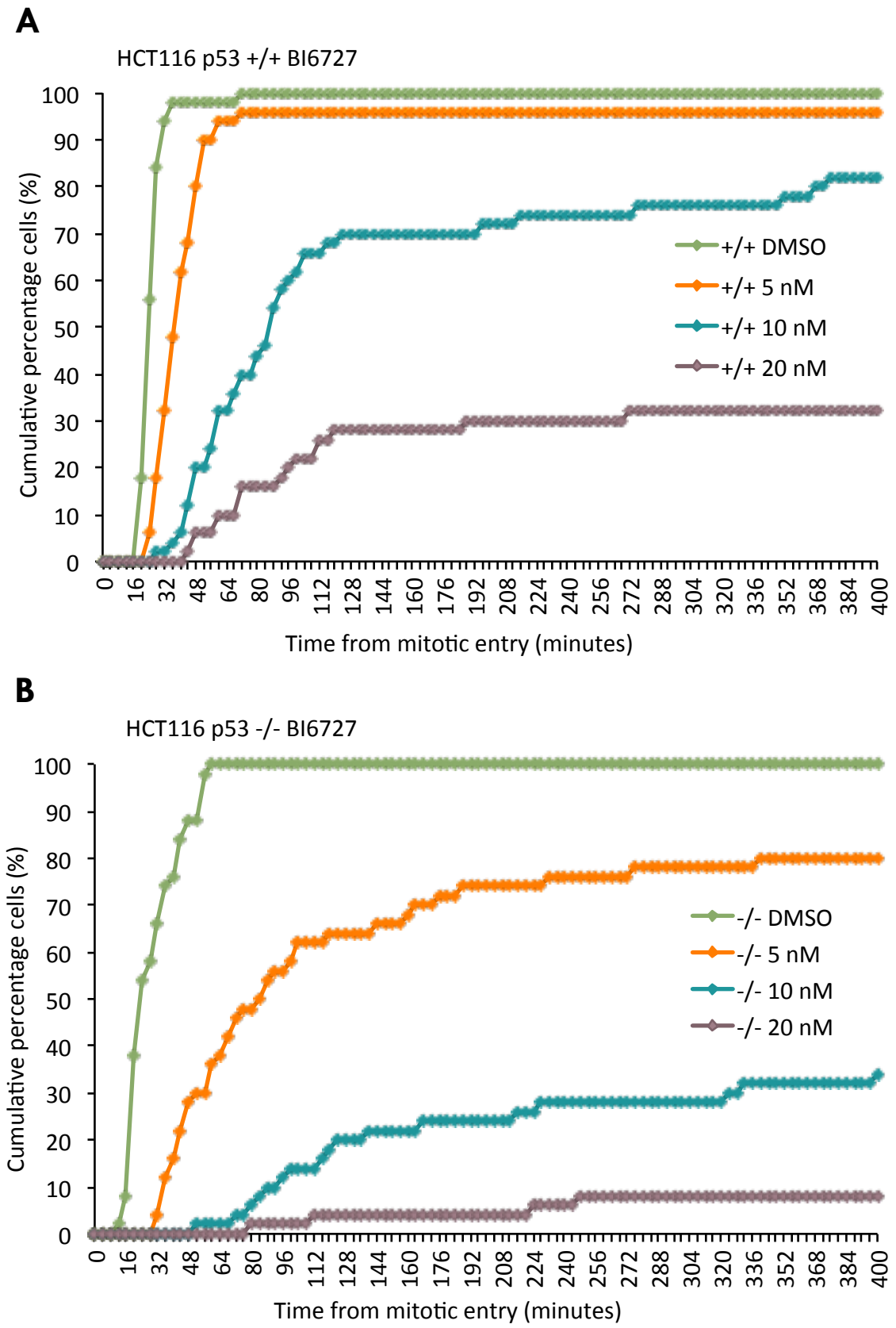


Figure 5.4. p53 reduces the duration of mitotic arrest upon treatment with BI6727. HCT116 p53+/+ (A) and p53-/- cells (B) were treated with 5, 10 or 20 nM BI6727 or DMSO as a vehicle control. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of three replicates.

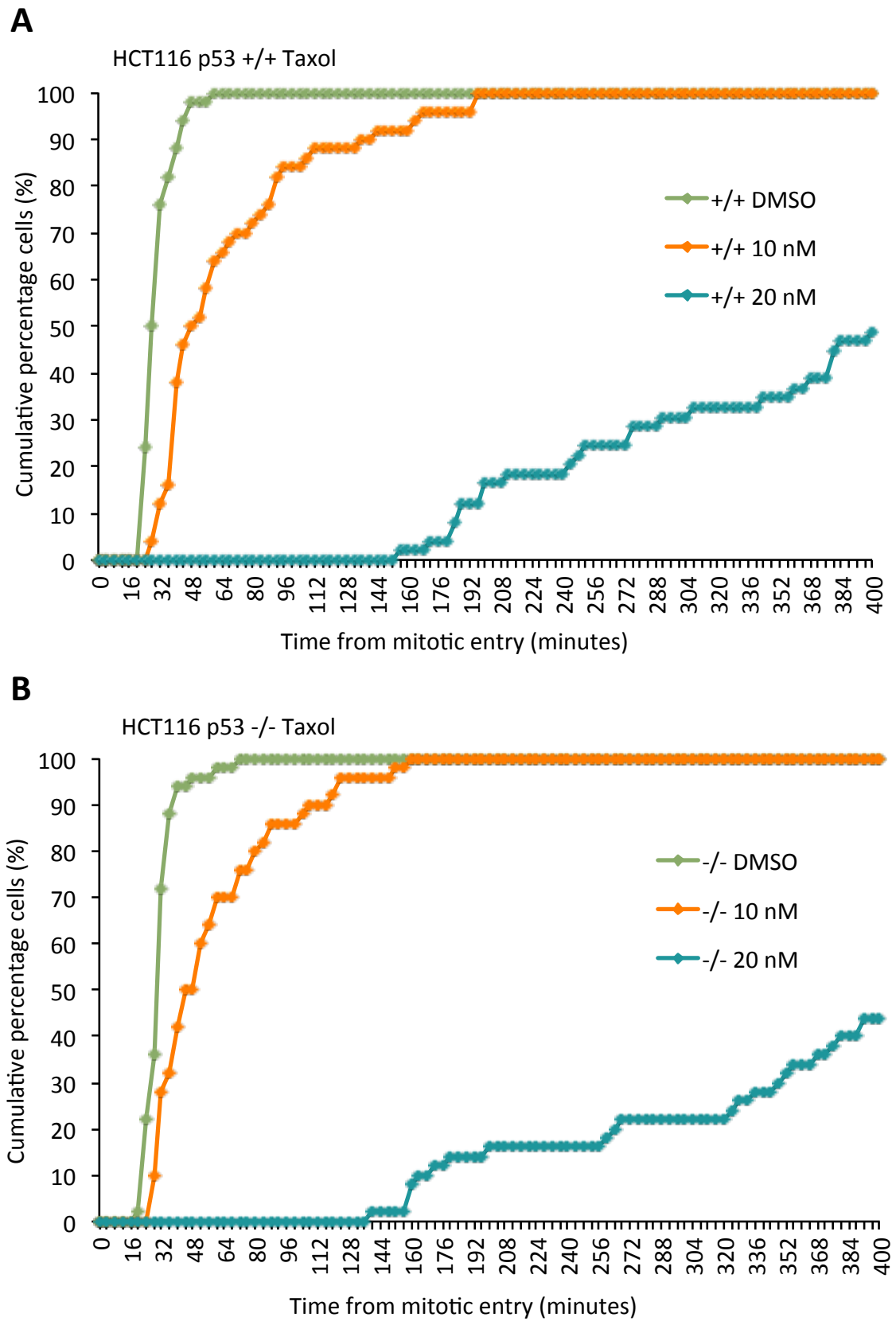


Figure 5.5. p53 has no effect on the duration of mitotic arrest upon treatment with Taxol. HCT116 p53+/+ (A) and p53-/- cells (B) were treated with 10 or 20 nM Taxol or DMSO as a vehicle control. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of two replicates.

To then determine if this effect was specific to p53 or just a cell line dependent effect, wild type MCF-7 (WT) cells and MCF-7 cells in which p53 has been knocked out by CRISPR (p53 KO) were tested. The first experiment tested the effect of GSK461364 on the MCF-7 WT and MCF-7 p53 KO cells. Figure 5.6 shows that under DMSO conditions the duration of mitosis is very similar between the WT and p53 KO cells. In both cases 100% of cells exited mitosis in the given time. As with the HCT116 cells, there was a dose dependent increase in the duration of mitotic arrest upon treatment with GSK461364 in both the WT and p53 KO cells. However, again the p53 deficient cells showed a more severe mitotic arrest in comparison to the p53 wild type cells at the equivalent doses of GSK461364.

As the MCF-7 cells showed a similar effect to the HCT116 cells upon inhibition of PLK1, the effects observed with treatment of Taxol were then tested. MCF-7 WT and p53 KO cells were treated with 20 or 40 nM Taxol or DMSO as a vehicle control. The resulting mitotic durations in Figure 5.7 showed that whilst increasing concentrations of Taxol resulted in an increase in the severity of the mitotic arrest, there was little difference between the MCF-7 WT and p53 KO cells. These findings support the results observed with the HCT116 cells, and suggest that the delay in mitotic exit upon treatment with PLK1 inhibitors is in fact dependent on the lack of p53 and not a cell line specific result.

To further enhance these experiments a similar approach was used, but this time to allow the DNA to be focused upon, SiR-DNA was used. HCT116 p53+/+ and p53-/- cells were seeded in specialised 8-well chambers and stained with 100 nM SiR-DNA. After staining the cells were treated with DMSO or 20 nM GSK461364. Cells were subsequently imaged using a Deltavision Elite microscope for 250 time points at 4 minutes intervals.

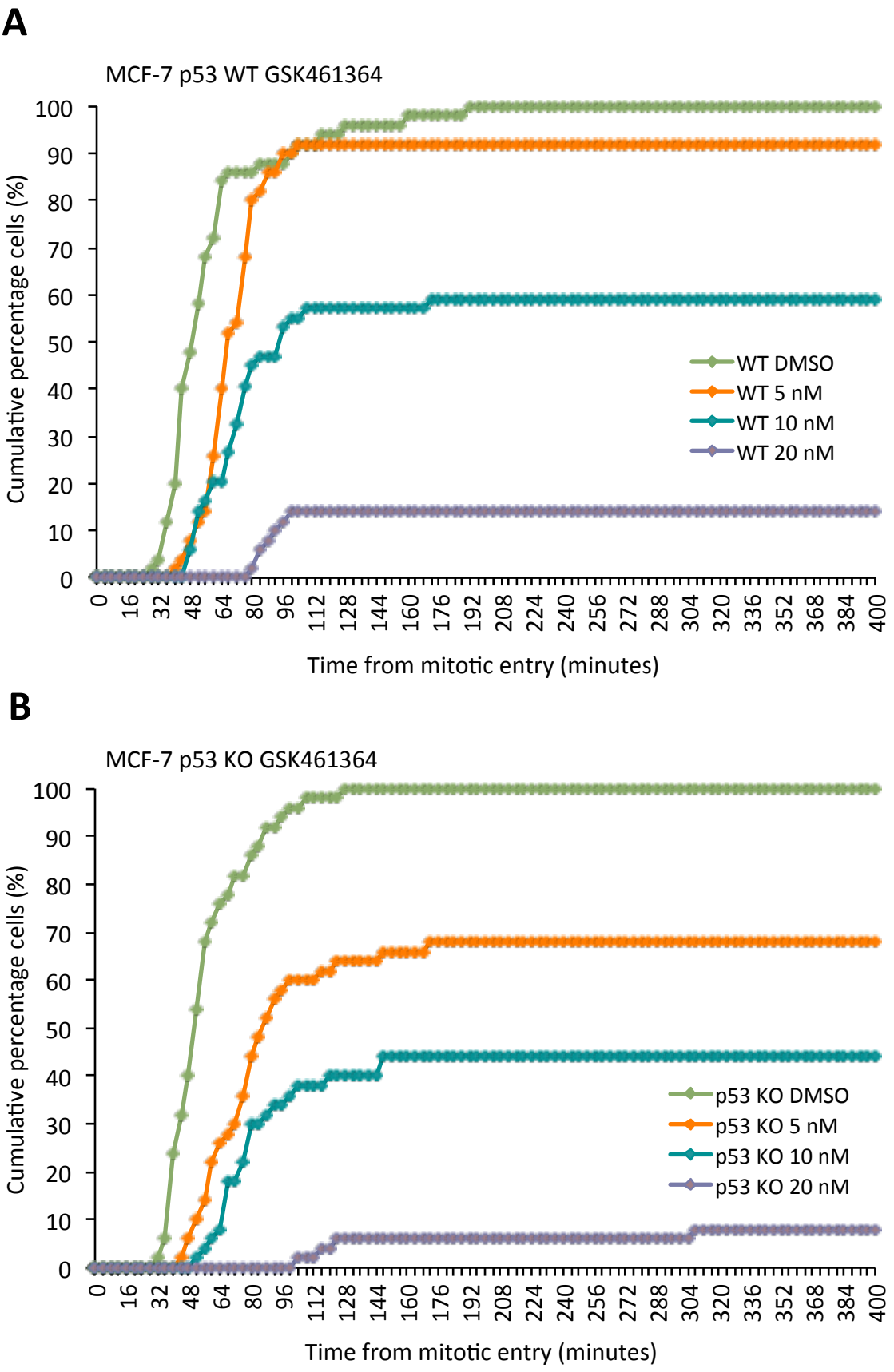


Figure 5.6. p53 reduces the duration of mitotic arrest upon treatment with GSK461364 in MCF-7 cells. MCF-7 wild type (WT) (A) and p53 KO cells (B) were treated with 5, 10 or 20 nM GSK461364 or DMSO as a vehicle control. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of two replicates.

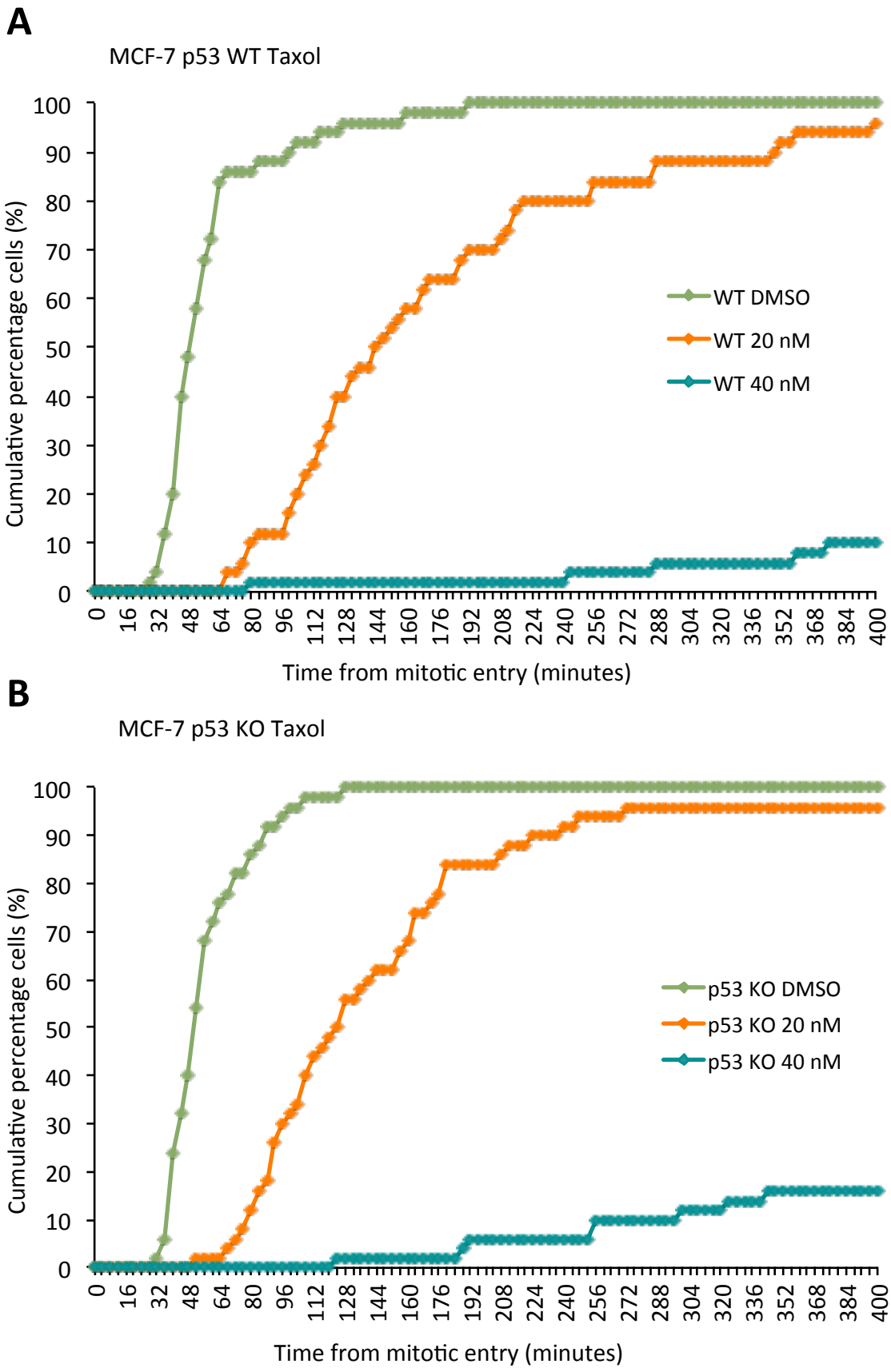


Figure 5.7. p53 has no effect on the duration of mitotic arrest upon treatment with Taxol in MCF-7 cells. MCF-7 WT (A) and p53 KO cells (B) were treated with 20 or 40 nM Taxol or DMSO as a vehicle control. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of two replicates.

The resulting movies, of which an electronic copy can be found in the appendix, allowed analysis of the DNA in the fluorescent channel and the cell in the phase contrast reference image. Representative images are shown in Figure 5.8 A, where a still at approximately 16 hours of treatment is shown. Whilst both the p53+/+ and p53-/- cells under DMSO conditions show few cells with condensed chromosomes (i.e mitotic cells), only the p53-/- cells show a much greater number of cells with condensed chromosomes upon treatment with GSK461364. Quantitative analysis of these movies is represented in Figure 5.8 B. Here it can be seen that under DMSO conditions the median duration of mitosis was very similar in the presence and absence of p53. However, when GSK461364 was applied the median duration of p53+/+ cells was 100 minutes, whilst the p53-/- cells had a median duration of 280 minutes. There was therefore a significant increase in the duration of mitosis in p53 deficient cells treated with GSK461364 compared to p53 competent cells. These findings support the data obtained by determining the duration of mitosis by use of phase contrast alone.

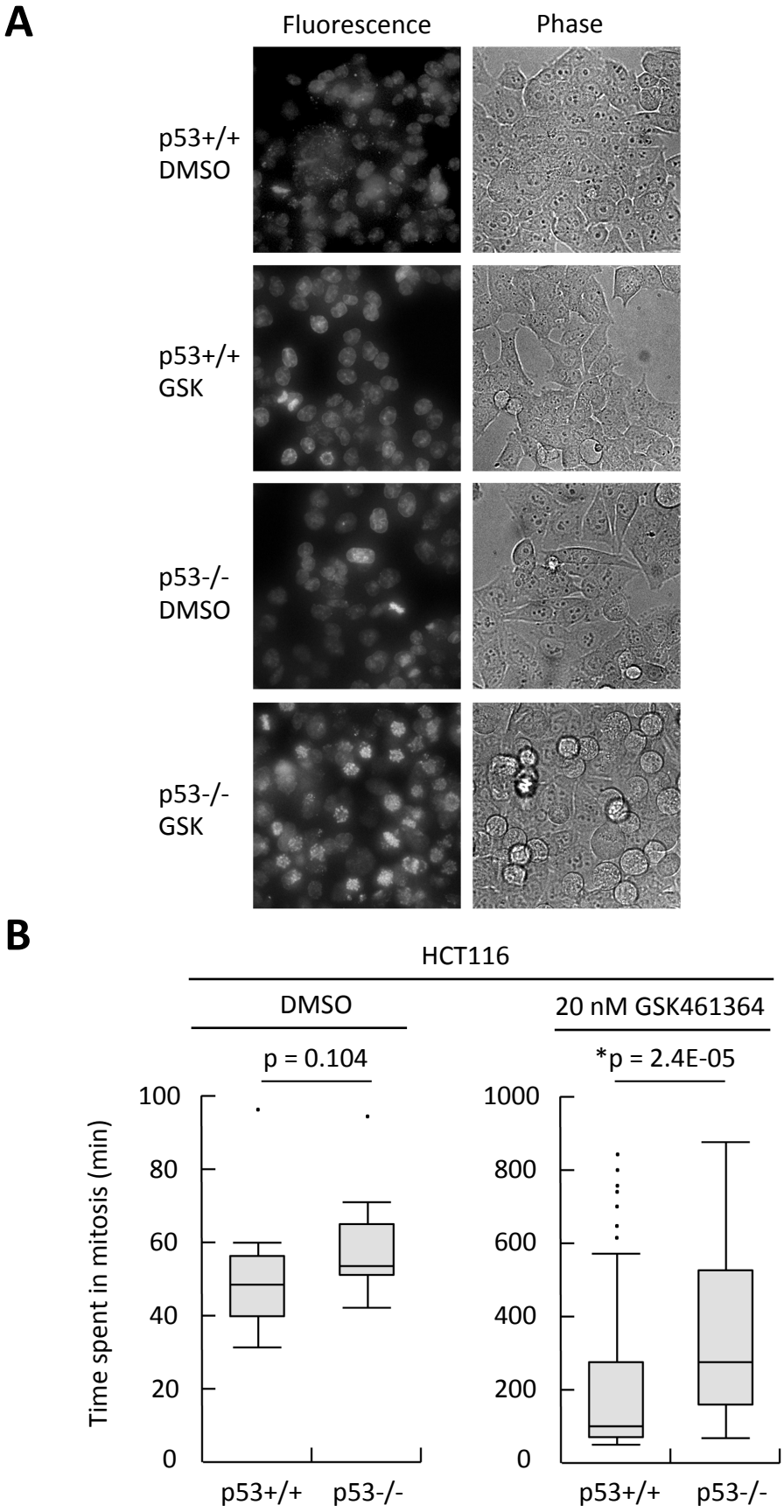


Figure 5.8. Upon inhibition of PLK1, p53 reduces the duration of mitotic delay. HCT116 p53+/+ and p53-/- cells were stained with SiR-DNA, treated with DMSO or 20 nM GSK461364 and imaged by time-lapse microscopy on a Deltavision Elite microscope. (A) Representative images of approximately 16 hours of treatment. (B) Box plots (showing median and quartiles) presenting the duration of mitosis of 22 HCT116 p53+/+ and 22 HCT116 p53-/- cells treated with DMSO and 77 HCT116 p53+/+ and 77 HCT116 p53-/- cells treated with 20 nM GSK461364. P values were calculated using a Student's t-test.

5.3.3. The presence of p53 results in an increased mitotic index upon inhibition of PLK1

To further investigate the mitotic arrest an alternative experimental procedure was used. Whilst normal cell cycle analysis does not discriminate G2 cells from mitotic cells, assays have been developed in order to quantify the mitotic index of a population of cells. Use of an antibody against phosphorylated histone H3 at serine 10, a site that is only phosphorylated during mitosis, allows calculation of the percentage of cells that are in mitosis at the time of harvesting. HCT116 p53+/+ and p53-/- cells were treated for 4 and 8 hours with 10 and 20 nM GSK461364. After fixation and incubation with first the phosphorylated histone H3 serine 10 antibody, and then the FITC secondary antibody, cells were stained with propidium iodide and analysed on the flow cytometer. The percentage of cells that were positive for phosphorylated histone H3 (serine 10), and therefore mitotic, were measured and plotted as a graph. The results represented in Figure 5.9 show that under DMSO conditions there is little difference between the mitotic index of the HCT116 p53+/+ and p53-/- cells. However, at both 10 and 20 nM GSK461364 and at both 4 (Figure 5.9 A) and 8 (Figure 5.9 B) hours of treatment the mitotic index is greater in the HCT116 p53-/- cells compared to the p53+/+ cells. This supports the findings from the experiments assessing the duration of mitotic arrest by time-lapse microscopy, where the p53-/- cells showed an increased duration of mitotic arrest compared to the p53+/+ cells. To check this was not specific to GSK461364, 10 and 20 nM BI6727 was also tested for 8 hours of treatment. Figure 5.9 C shows that again, at both concentrations of BI6727 the p53-/- cells have an increased mitotic index in comparison to the p53+/+ cells.

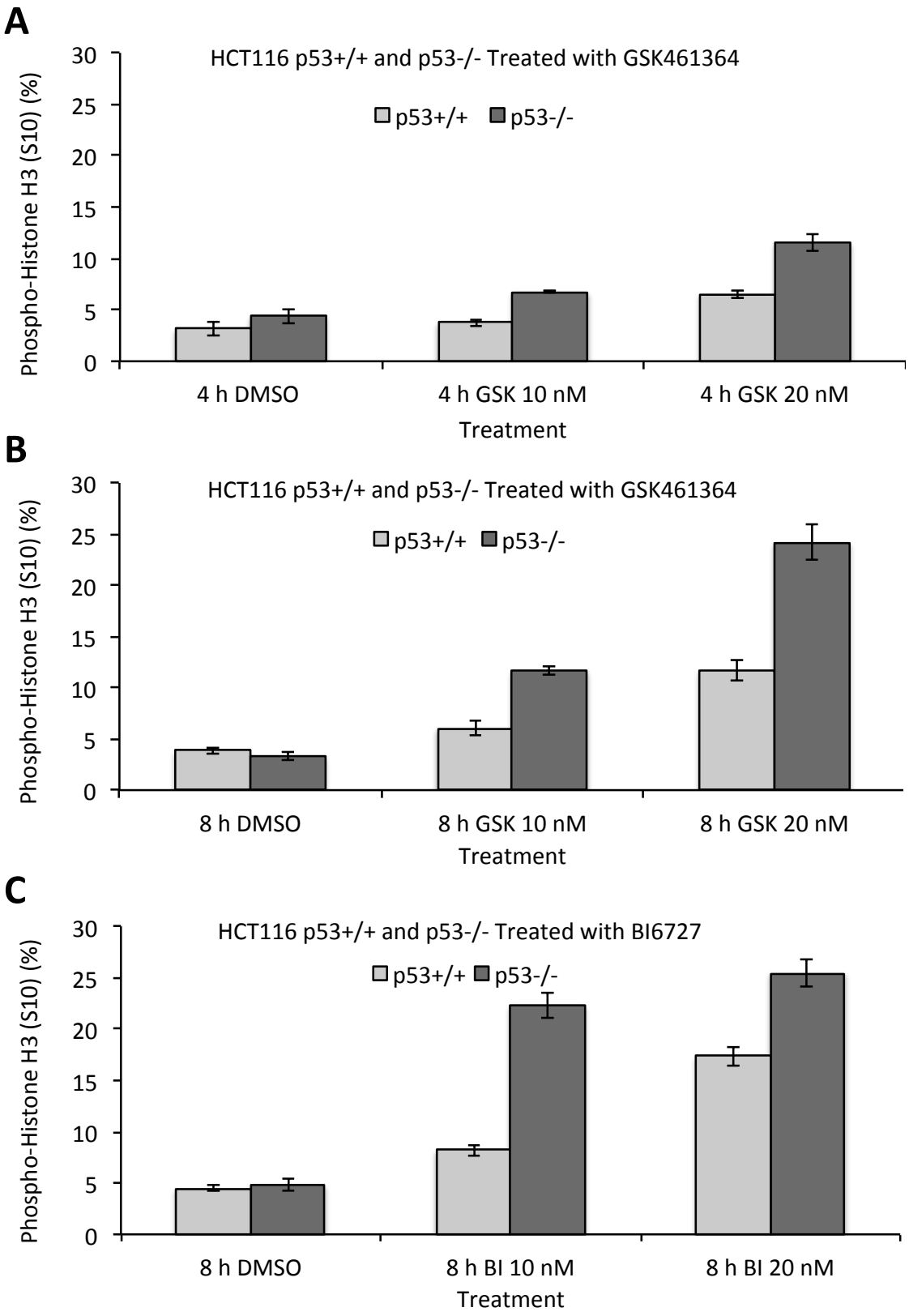


Figure 5.9. HCT116 p53-/- cells show an increased mitotic index upon treatment with PLK1 inhibitors in comparison to HCT116 p53+/+ cells. HCT116 p53+/+ and p53-/- cells were treated with 10 nM or 20 nM GSK461364 (A+B) or BI6727 (C) for 4 hours (A) or 8 hours (B+C). Cells were subsequently harvested, fixed, stained with phosphorylated histone H3 (serine 10) antibody, followed by staining with a FITC secondary antibody, and finally labelled with propidium iodide. Flow cytometry was then used to calculate the percentage of cells that were positive for phospho-histone H3 (S10) and therefore mitotic. The graphs are representative of two independent experiments.

5.3.4. Inhibition of DNA damage-induced activation of p53 upon PLK1 inhibition increases the duration of mitotic arrest

The next aim was to determine how p53 results in a quicker decision in mitosis in the presence of PLK1 inhibitors. Previously, it had been shown that the protein kinases ATM and ATR were involved in the activation of p53 upon inhibition of PLK1. Therefore, to test if the activation of p53 by the DNA damage response is required for the observed differences in mitotic duration, the assay measuring the mitotic duration was repeated in the presence or absence of ATM and ATR inhibitors. Figure 5.10 shows that in comparison to the DMSO control, addition of ATM and ATR inhibitors (KU-55933 and VE-821) alone resulted in a slight delay in mitosis. However, the vast majority of cells were exiting mitosis in the time frame analysed. Treatment with GSK461364 resulted in an increased mitotic duration as previously observed. Pretreatment with ATM and ATR inhibitors followed by treatment with GSK461364 in the HCT116 p53+/+ line resulted in an increase in the severity of the mitotic arrest in comparison to GSK461364 treatment alone. However, in the HCT116 p53-/- line GSK461364 treatment alone or in combination with ATM and ATR inhibitors showed virtually indistinguishable differences in mitotic duration. This suggested that the DNA damage response leading to activation of p53 is, at least in part, required to allow a quicker mitotic duration in the presence of PLK1 inhibitors. The same experiment was carried out with BI6727 in place of GSK461364. Figure 5.11 shows that a very similar result was obtained.

These observations suggest that p53 does require activation via the DNA damage response pathways for this mitotic effect to occur. However, it doesn't distinguish between the possibility that p53 is actively involved during this stage of mitosis or if the initial delay actually arises from events that occur prior to mitosis.

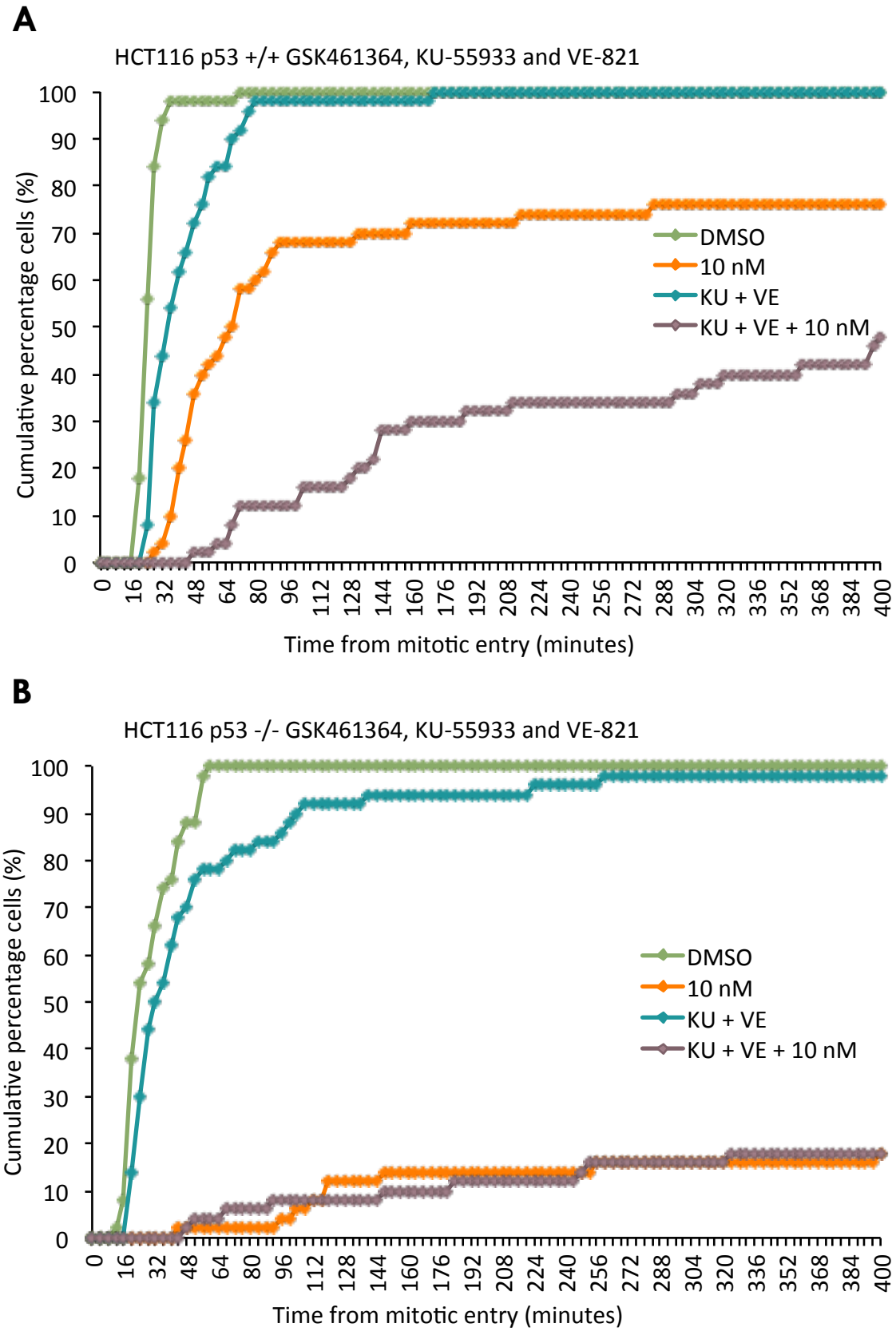


Figure 5.10. Mitotic effect of inhibiting the DNA damage response upon treatment with GSK461364. HCT116 p53+/+ (A) and p53-/- (B) cells were pretreated for 1 hour with DMSO or 10 μM ATM and ATR inhibitors, KU-55933 and VE-821 respectively. Cells were subsequently treated with DMSO or 10 nM GSK461364. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of two replicates.

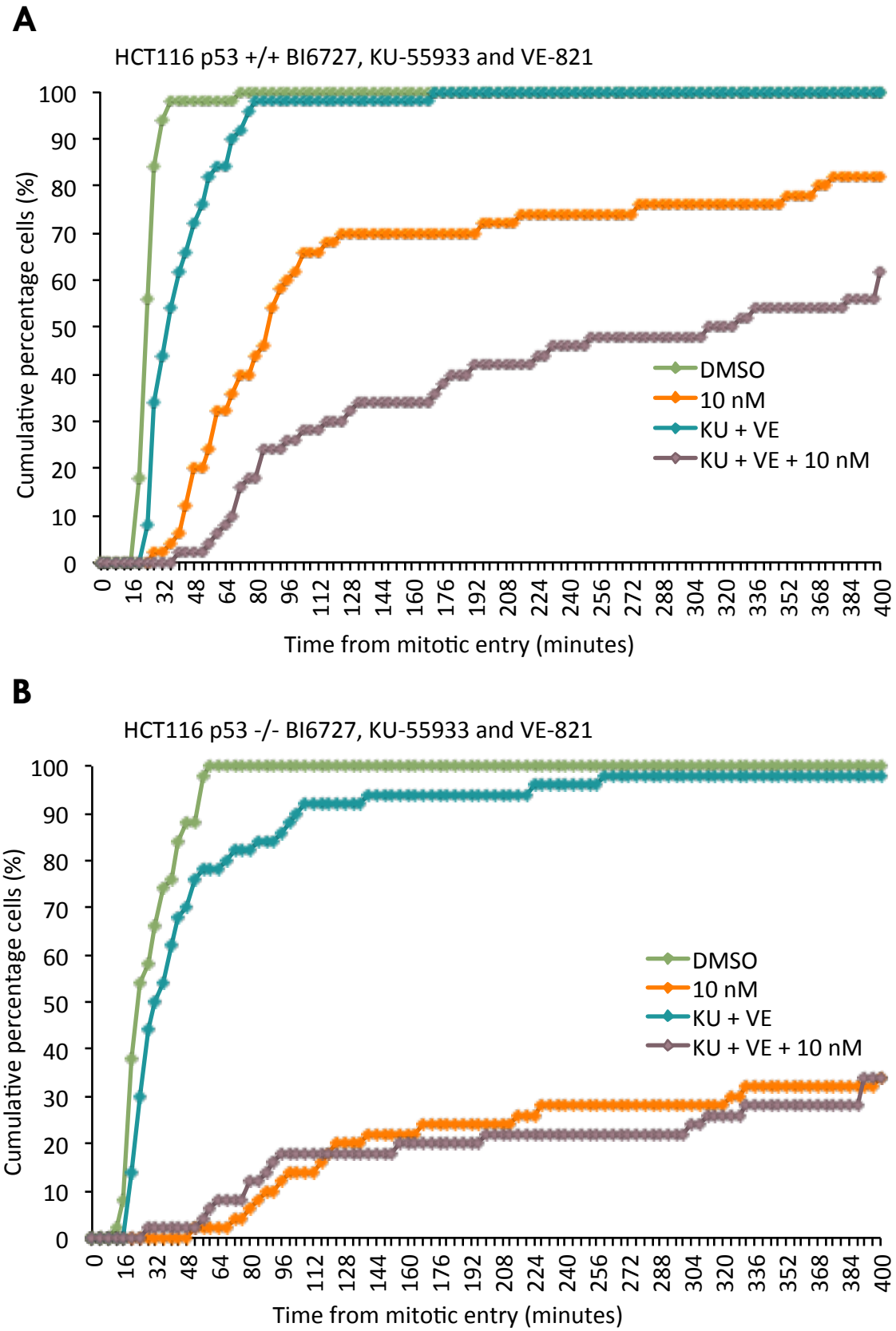
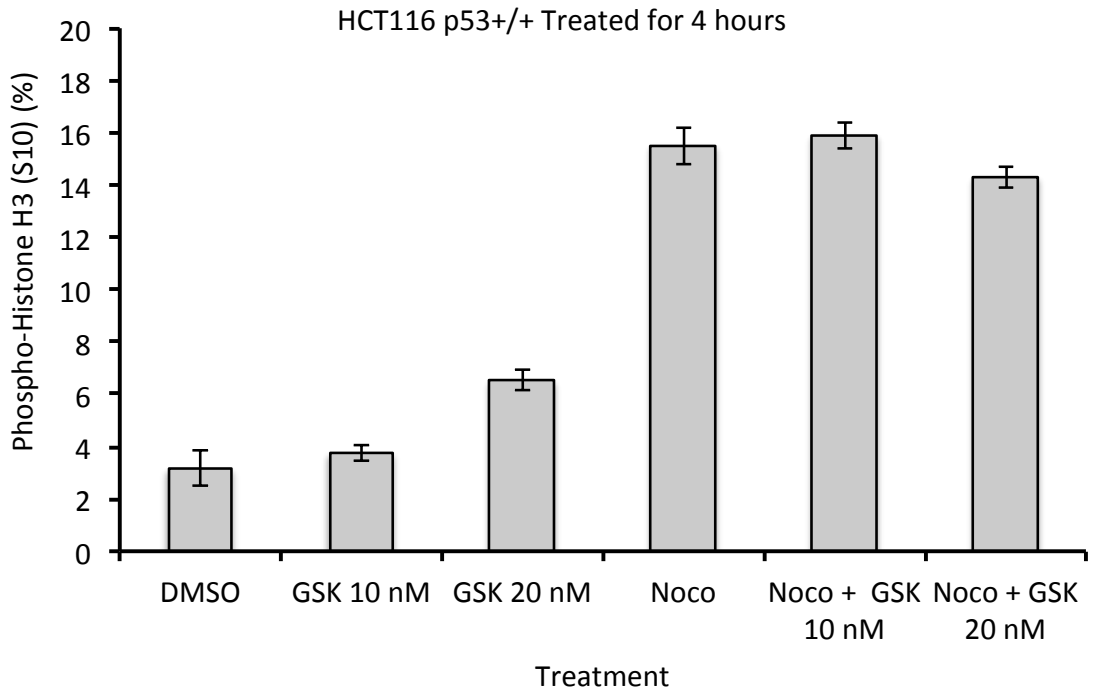


Figure 5.11. Mitotic effect of inhibiting the DNA damage response upon treatment with BI6727. HCT116 p53+/+ (A) and p53-/- (B) cells were pretreated for 1 hour with DMSO or 10 μM ATM and ATR inhibitors, KU-55933 and VE-821 respectively. Cells were subsequently treated with DMSO or 10 nM BI6727. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of two replicates.

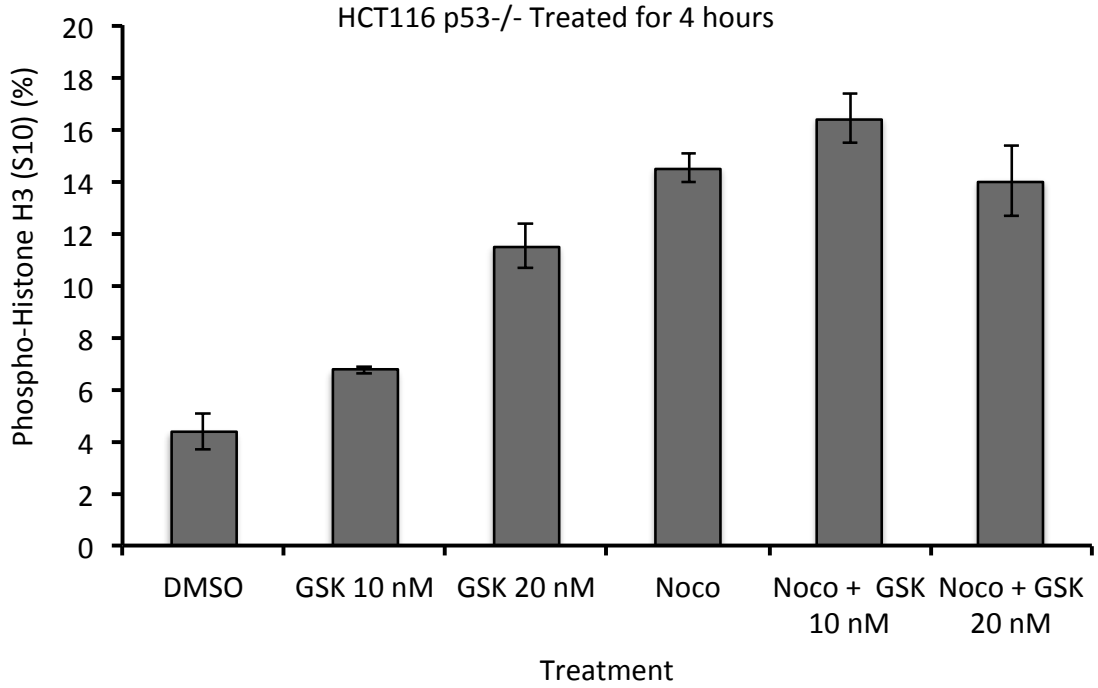
5.3.5. p53 does not cause a delay in mitotic entry upon inhibition of PLK1

In order to determine if cells traverse mitosis more successfully in the presence of p53 due to a delay in mitotic entry, allowing cells to somehow prepare prior to entry to mitosis and resulting in a more successful chance of completing mitosis, flow cytometry was used. To give a measure of the number of cells entering mitosis it was necessary to block mitotic exit, thereby allowing the accumulation of cells to represent a measure of mitotic entry rate. Nocodazole, a mitotic inhibitor that interferes with the polymerisation of microtubules, was used in order to prevent cells exiting mitosis. Therefore, with use of Nocodazole and/or PLK1 inhibitor the effect of PLK1 inhibition on mitotic entry could be assessed. HCT116 p53+/+ and p53-/- cells were treated with DMSO, 10 nM GSK461364, 20 nM GSK461364, Nocodazole, Nocodazole and 10 nM GSK461364 or Nocodazole and 20 nM GSK461364 for 4 and 8 hours. Flow cytometry was then used to analyse the percentage of cells in mitosis by detecting cells that were positive for phospho-histone H3 (S10). Figure 5.12 shows that upon treatment with 10 nM or 20 nM GSK461364 the mitotic index is increased compared to the DMSO control. Treatment with Nocodazole alone resulted in a high percentage of mitotic cells, showing the cells were being prevented from exiting mitosis in both the p53+/+ and p53-/- lines. In combination with Nocodazole, GSK461364 had little effect on the proportion of cells in mitosis. This therefore suggested that cells were not being delayed in their entry to mitosis upon inhibition of PLK1, as in this case the percentage of mitotic cells would decrease in combination of Nocodazole and GSK461364 compared to Nocadozole alone.

A


HCT116 p53+/+ Treated for 4 hours
Phospho-Histone H3 (S10) (%)
0
2
4
6
8
10
12
14
16
18
20
DMSO
GSK 10 nM
GSK 20 nM
Noco
Noco + GSK 10 nM
Noco + GSK 20 nM
Treatment

B


HCT116 p53-/- Treated for 4 hours
Phospho-Histone H3 (S10) (%)
0
2
4
6
8
10
12
14
16
18
20
DMSO
GSK 10 nM
GSK 20 nM
Noco
Noco + GSK 10 nM
Noco + GSK 20 nM
Treatment

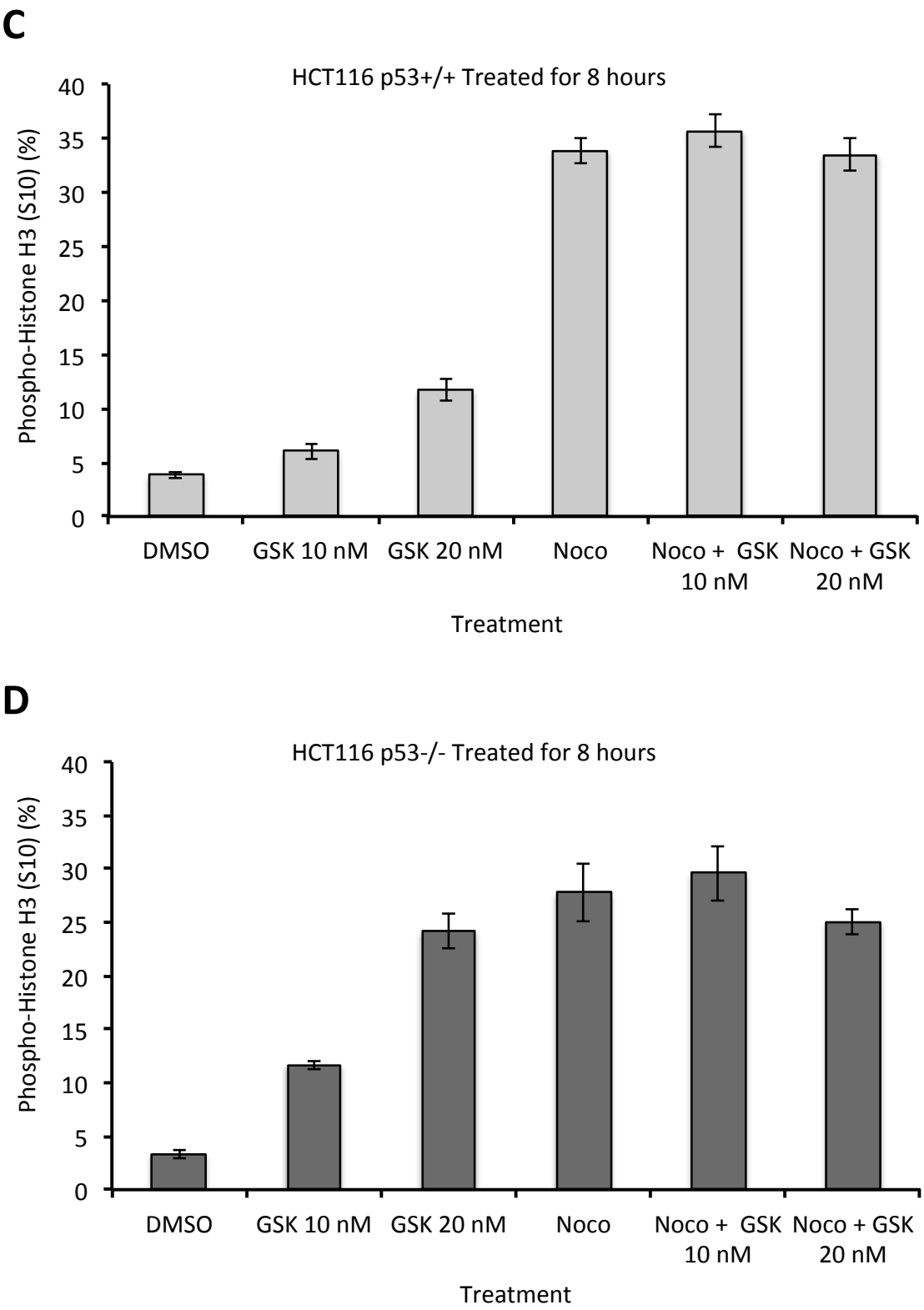


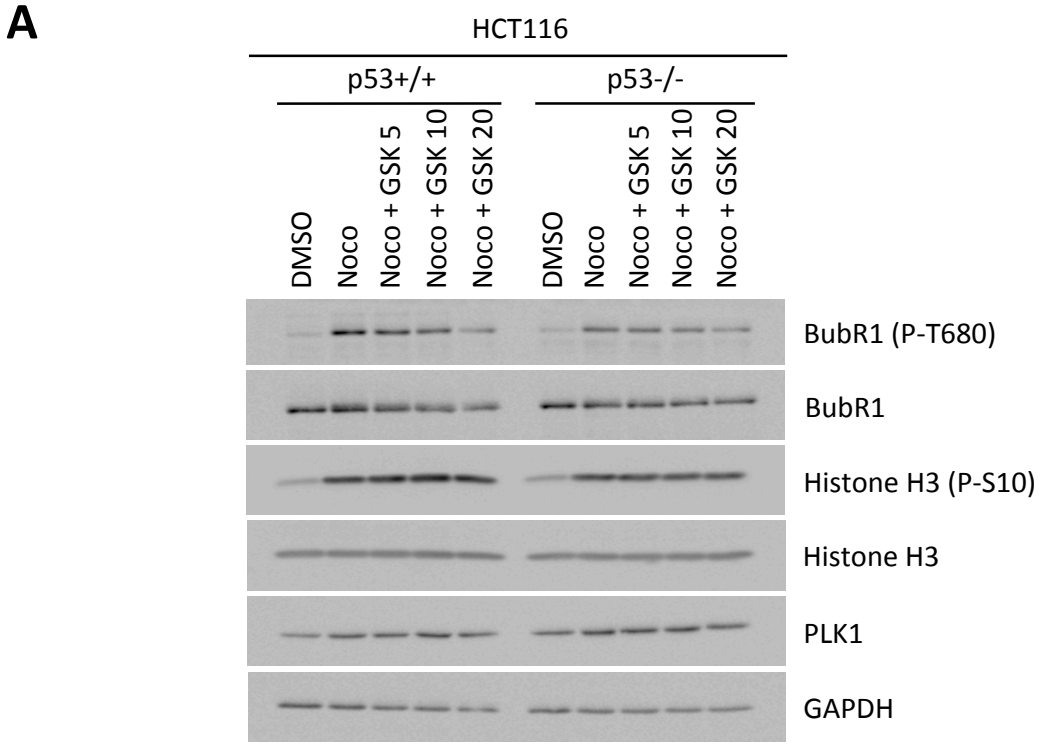
Figure 5.12. There is not a p53 dependent delay in mitotic entry upon treatment with GSK461364. HCT116 p53+/+ (A+C) and p53-/- (B+D) cells were treated with 10 nM or 20 nM GSK461364, 3.3 μM Nocodazole or a combination of 10 nM or 20 nM GSK461364 with 3.3 μM Nocodazole for 4 hours (A+B) or 8 hours (C+D). Cells were subsequently harvested, fixed, stained with phosphorylated histone H3 (serine 10) antibody, followed by staining with a FITC secondary antibody, and finally labelled with propidium iodide. Flow cytometry was then used to calculate the percentage of cells that were positive for phospho-histone H3 (S10) and therefore mitotic. The graphs are representative of two independent experiments.

5.3.6. There is not a p53-dependent difference in PLK1 activity upon inhibition of PLK1

As there was not a p53-dependent delay in mitotic entry observed when PLK1 inhibitors were applied, perhaps the activity of PLK1 was higher in HCT116 p53+/+ cells compared to p53-/- cells upon inhibition of PLK1. This could allow p53 competent cells to complete mitosis due to having sufficient levels of PLK1 to allow mitotic progression. To test this hypothesis a phosphorylation target of PLK1, threonine 680 in BubR1, was measured (Suijkerbuijk *et al.*, 2012). As PLK1 is most active in mitosis, and upon inhibition of PLK1 there are many more cells in mitosis in the HCT116 p53-/- line compared to the p53+/+ line, the differences in the number of mitotic cells had to be considered. In order to circumvent this problem, cells were treated with Nocodazole, or Nocodazole in combination with PLK1 inhibitors. As the flow cytometry data (Figure 5.12) showed that the number of mitotic cells was very similar when Nocodazole was used alone or in combination with GSK461364, this provided a useful means by which to assess the PLK1 activity. Cells were therefore treated with DMSO, Nocodazole, Nocodazole and 5 nM GSK461364, Nocodazole and 10 nM GSK461364 or Nocodazole and 20 nM GSK461364 for 4 or 8 hours.

Figure 5.13 shows that as expected the levels of phosphorylated histone H3 at serine 10 are increased in the Nocodazole and Nocodazole/ GSK461364 combination treatments compared to the DMSO control. The levels of phosphorylated histone H3 (S10) were fairly consistent throughout the different combinations of drug treatments. The phosphorylation of BubR1 showed that the levels decrease with increasing concentrations of GSK461364. However, this is the case independent of p53 status. There is therefore not a significant difference in PLK1 activity between the p53+/+ and p53-/- cells. This suggests the activity of PLK1 alone, does not account for the

differences observed between the two lines in regards to the assessments of mitotic events.



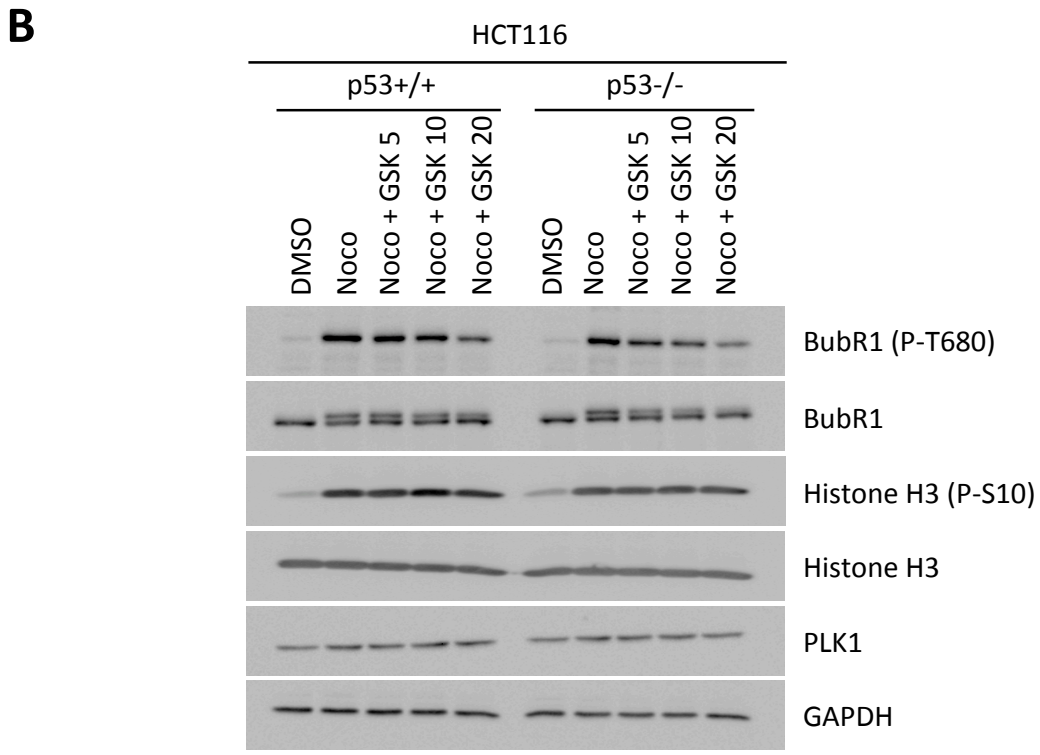


Figure 5.13. PLK1 activity in HCT116 p53+/+ and p53-/- cells upon treatment with PLK1 inhibitors. HCT116 p53+/+ and p53-/- cells were treated with DMSO, 3.3 µM Nocodazole, or 3.3 µM Nocodazole in combination with 5, 10 or 20 nM GSK461364 for 4 hours (A) or 8 hours (B). Cells were subsequently harvested and western blotting was performed with the resulting cell lysates.

5.3.7. p53 plays a role in centrosome separation upon inhibition of PLK1

Once entry into mitosis is achieved, PLK1 is involved in the maturation and separation of centrosomes. The characteristic 'polo' arrest results as a consequence of the centrosomes failing to separate and migrate to opposite sides of the cell. This results in a monopolar spindle rather than the bipolar spindle required for successful separation of the chromatid pairs. Studies have linked p53 to the centrosomes for several years, both in terms of localisation and functions. The functions of p53 at the centrosomes are regulation of centrosome duplication and regulation of centrosome separation (Fukasawa *et al.*, 1996; Tritarelli *et al.*, 2004; Nam and van Deursen, 2014). Therefore to determine if the presence of p53 results in more successful separation of centrosomes upon PLK1 inhibition, compared to the absence of p53, immunofluorescence with centrosome staining was used. Cells were fixed and stained with a γ-tubulin antibody, a marker for the centrosome, and then Alexa-fluor secondary antibody and DAPI. The centrosomes were then visualised in mitotic cells and the number of cells with separated centrosomes versus non-separated centrosomes were counted. The resulting numbers under DMSO or PLK1 inhibitor treated samples can be seen in Figure 5.14 A. As can be seen, under DMSO conditions separation of centrosomes in mitotic cells occurs in nearly all the cells analysed and there is little difference between the p53+/+ and p53-/- cells. Upon treatment with GSK461364 the number of separated centrosomes in mitotic cells is significantly reduced in both the p53+/+ and p53-/- cells compared to the DMSO controls. However, the number of separated centrosomes is much higher than the non-separated in the p53+/+ cells, whereas in the p53-/- cells the converse is true. Figure 5.14 B shows representative images of the staining.

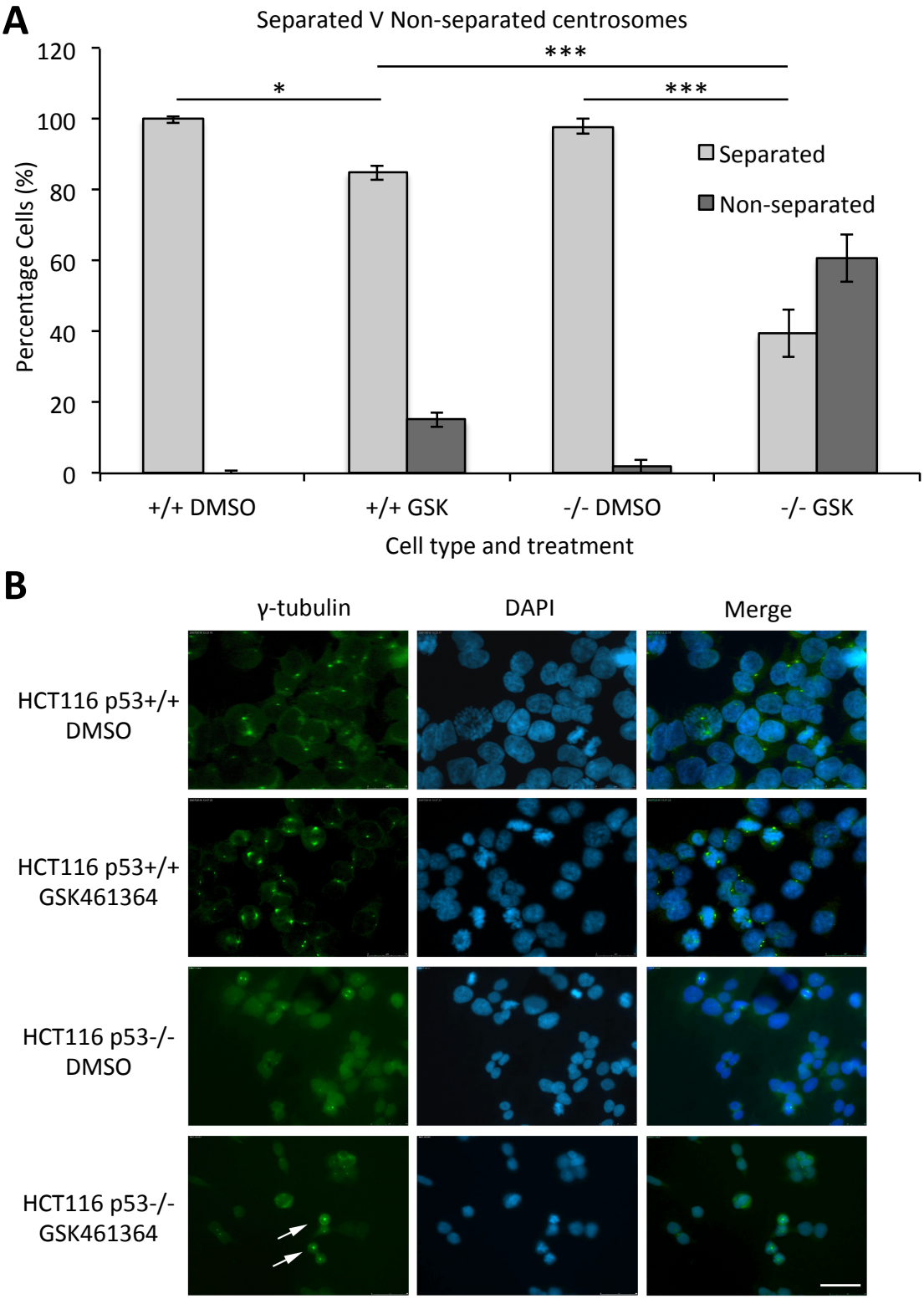


Figure 5.14. Centrosome separation in HCT116 cells upon treatment with GSK461364. HCT116 p53+/+ and p53-/- cells were treated with 20 nM GSK461364 or DMSO for 8 hours. Cells were subsequently fixed and stained with γ-tubulin antibody (a centrosome marker), followed by secondary staining with Alexa Fluor® 488 and DAPI. Fluorescence microscopy was then used to allow manual determination of the number of mitotic cells showing a normal bipolar spindle (separated centrosomes) or monopolar spindle (non-separated centrosomes). Over 100 cells were counted for each condition (scored by two independent individuals) for each repeat and the average percentages of separated v non-separated centrosomes were calculated and plotted as a bar graph. Statistical significance is determined by *$p<0.05$ and ***$p<0.001$ (A). Representative images for each condition are shown in (B). White arrows indicate the non-separated centrosomes in mitotic cells. The scale bar represents 75 microns.

5.3.8. p53 influences the pathway involved in centrosome separation

Due to the observed differences in centrosome separation between the p53+/+ and p53-/- cells upon treatment with PLK1 inhibitors, the next aim was to investigate the pathway involved in centrosome separation. Centrosome separation is a two-step process, with the linker firstly being broken, and then a motor protein physically driving the centrosomes apart. PLK1 is involved in both of these steps. However, the motor protein required for the separation, Eg5, has inhibitors available, therefore allowing manipulation of the pathway. It is known that inhibition of Eg5 causes a similar mitotic arrest to inhibition of PLK1, with formation of a monopolar spindle. Therefore, to test if the differences in centrosome separation between the p53+/+ and p53-/- cells was dependent on PLK1 or a consequence of impairment of the pathway, STLC, an Eg5 inhibitor was used. STLC targets the catalytic domain of Eg5 and inhibits both the basal and microtubule-activated ATPase activity of Eg5 (Skoufias *et al.,* 2006). If the differences occurred between p53+/+ and p53-/- cells under inhibition of Eg5, this would suggest that the centrosomal pathway is affected by p53. HCT116 p53+/+ and p53-/- cells were treated with a range of doses of STLC and imaged over 16 hours at four minutes intervals. Analysis of the mitotic duration was carried out and as can be seen in Figure 5.15 use of the Eg5 inhibitor results in a similar phenotype to the PLK1 inhibitors, with p53-/- cells showing a more severe mitotic arrest compared to the p53+/+ cells. This result is consistent with the idea that PLK1 is responsible for Eg5 activation and that PLK1 inhibition would impair Eg5 activation.

To then determine if inhibition of Eg5 showed p53-dependent sensitivity similar to that seen with the PLK1 inhibitors, an MTS assay was carried out with the HCT116 p53+/+ and p53-/- cells with a range of concentrations of STLC. Figure 5.16 A shows that the results mirror inhibition of PLK1, with increased comparative sensitivity to STLC in the

p53-/- cell line. Additionally, western blotting was carried out, with treatments of 24, 48 or 72 hours. As can be seen in Figure 5.16 B the PLK1 levels throughout the treatment time course, show similar levels to that when PLK1 inhibitors are applied, with an increase in abundance of PLK1 at 24 hours of treatment. Similarly, p53 and p21 levels increase with treatment, and DNA damage occurs as shown by phosphorylated H2AX. This strongly suggests that the effects observed with PLK1 inhibitors are due to impairment of the centrosome separation pathway of which Eg5 is within.

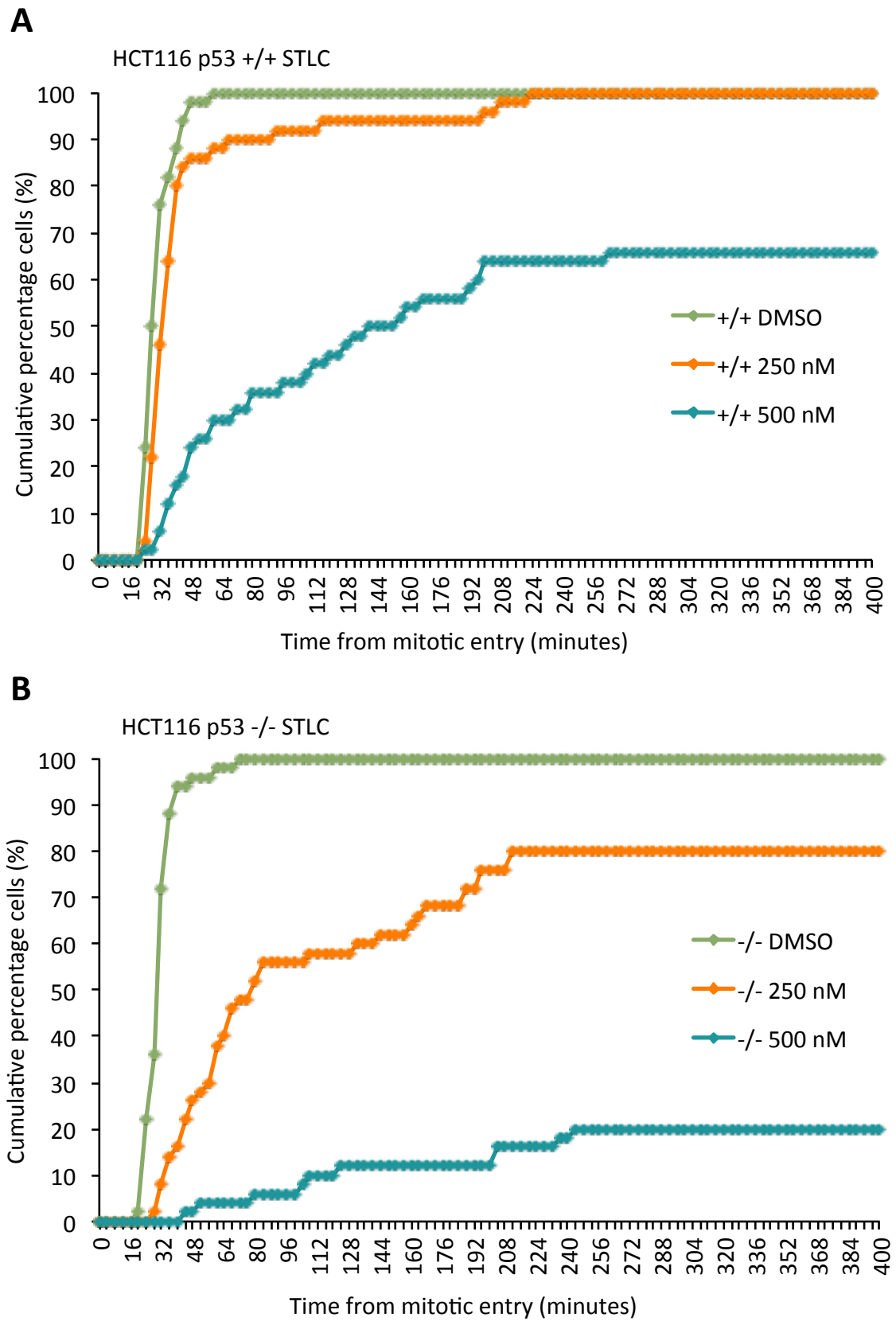


Figure 5.15. p53 reduces the duration of mitotic arrest upon treatment with STLC. HCT116 p53+/+ (A) and p53-/- cells (B) were treated with 250 or 500 nM STLC or DMSO as a vehicle control. Time-lapse analysis was then used to determine the duration of mitosis. Both graphs represent the cumulative data from 50 cells for one experiment and are representative of two replicates.

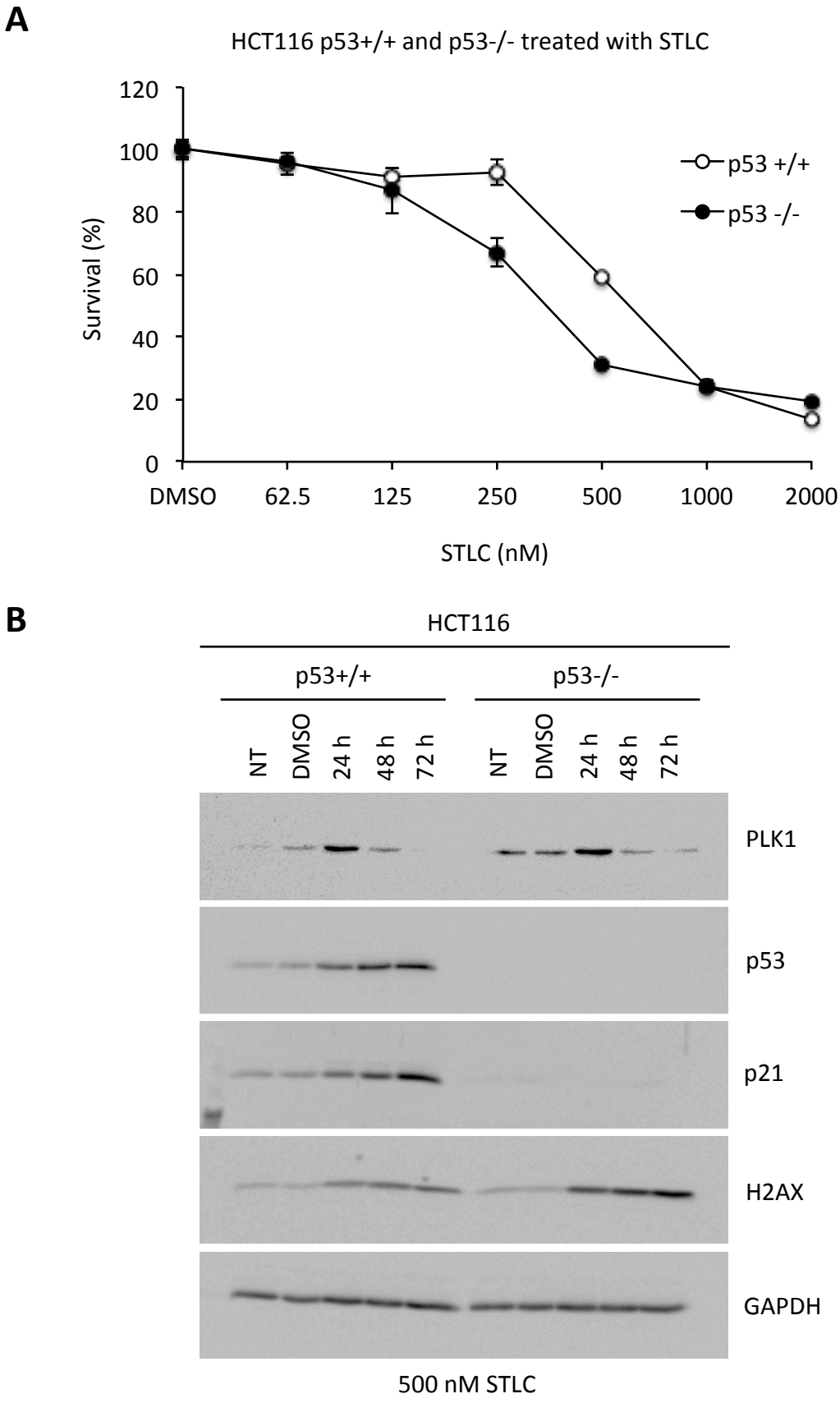


Figure 5.16. HCT116 p53+/+ and p53-/- cells treated with STLC. (A) HCT116 p53+/+ and p53-/- cells were treated with DMSO or 62.5, 125, 250, 500, 1000 or 2000 nM STLC. After 72 hours of treatment an MTS assay was used to determine cell survival. (B) HCT116 p53+/+ and p53-/- cells were treated with 500 nM STLC for 24, 48 or 72 hours. Western blotting was then used with the antibodies indicated in the figure.

For these differences to emerge, p53 would either have to directly act on Eg5, or act on a pathway that can compensate for loss of Eg5. Studies have shown that Kif15, a functionally redundant motor protein of Eg5, can separate the centrosomes in the absence of Eg5 (Tanenbaum *et al.*, 2009). Therefore, it was hypothesised that p53 may have an effect on Eg5 or the pathway involving Kif15. Therefore, the levels of Eg5 and Kif15 proteins were assessed in the HCT116 p53+/+ and p53-/- cells and the MCF-7 wild type and p53 KO cells under basal conditions. Figure 5.17 shows that in both HCT116 and MCF-7, cells the levels of Kif15 appear to be slightly lower in the absence of p53, whilst Eg5 levels appeared to be p53-independent.

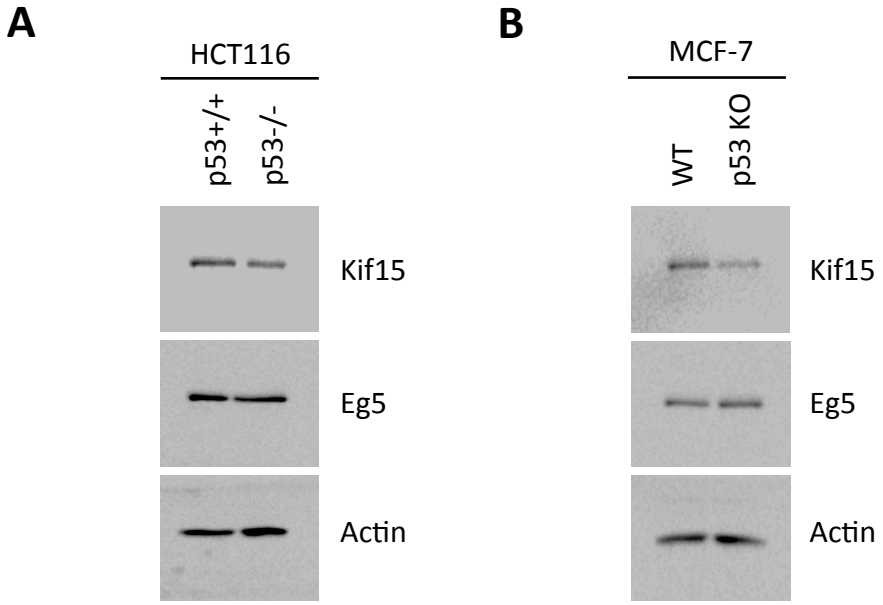


Figure 5.17. Levels of Kif15 and Eg5 in p53 competent and p53 knockout cells. Untreated HCT116 p53+/+ and p53-/- (A) and MCF-7 wild type and p53 knockout cells (B) were harvested and the cell lysate was analysed by western blotting.

As a result of the observation that the presence of p53 may affect the levels of Kif15, the next aim was to determine if either Kif15 or Eg5 are p53 inducible. In order to test this, the first experimental approach was to treat cells with the MDM2 inhibitor, Nutlin-3, for up to six hours to induce a p53 response and assess whether the levels of Kif15 or Eg5 increased. As can be seen in Figure 5.18 whilst Nutlin-3 caused an induction in p53, there was no corresponding p53-dependent increase in either Kif15 or Eg5 levels.

Whilst Nutlin-3 results in induction of p53, this occurs by inhibiting MDM2 and thus preventing the degradation of p53. Therefore, no DNA damage occurs, so we hypothesised that p53 may have to be induced by DNA damage to result in any observable differences in Kif15 or Eg5 levels. Two concentrations of etoposide were tested for six and sixteen hours of treatment. Figure 5.19 shows that again, as with Nutlin-3, there were no p53-dependent increases in Eg5 or Kif15 levels. Whilst Eg5 levels fluctuated with treatment, this was not in a p53-dependent manner.

At this point it seemed unlikely that Kif15 or Eg5 were p53 targets, either directly or indirectly. However, the p53-dependent differences that had been observed at the centrosomes occurred under inhibition of PLK1 or Eg5. As a final check, the Eg5 and Kif15 levels were tested under treatment with PLK1 inhibitors, GSK461364 or BI6727, and Eg5 inhibitor, STLC. Figure 5.20 shows that under all treatment types, in both the HCT116 p53+/+ and p53-/- and MCF-7 wild type and p53 knockout cells, the changes in Eg5 levels were independent of p53. Whilst the Kif15 levels appeared higher in the p53 competent lines, there was no obvious induction upon treatment with either PLK1 or Eg5 inhibitors.

Overall, these findings suggest p53 does not affect Eg5 or Kif15 levels upon treatment with a variety of drugs. Therefore, it seems unlikely that Kif15 is substituting for Eg5 in

a p53-dependent manner when the pathway involved in centrosome separation is inhibited.

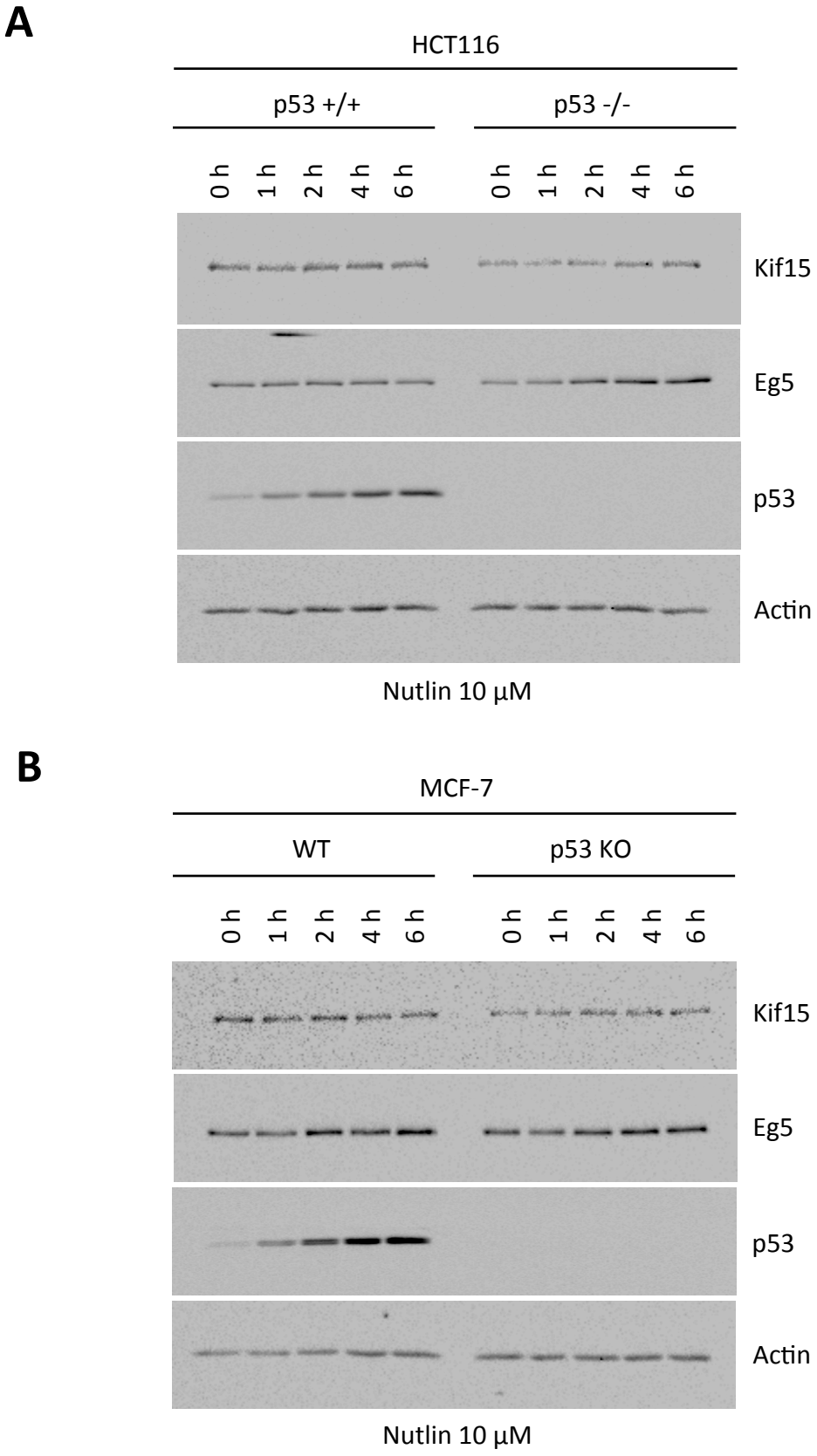


Figure 5.18. Kif15 and Eg5 levels upon treatment with Nutlin-3. HCT116 p53+/+ and p53-/- cells (A) or MCF-7 wild type and p53 knockout cells (B) were treated with 10 µM Nutlin-3 for 0, 1, 2, 4 or 6 hours. Cells were subsequently harvested, and analysed by western blotting with the antibodies indicated in the figure.

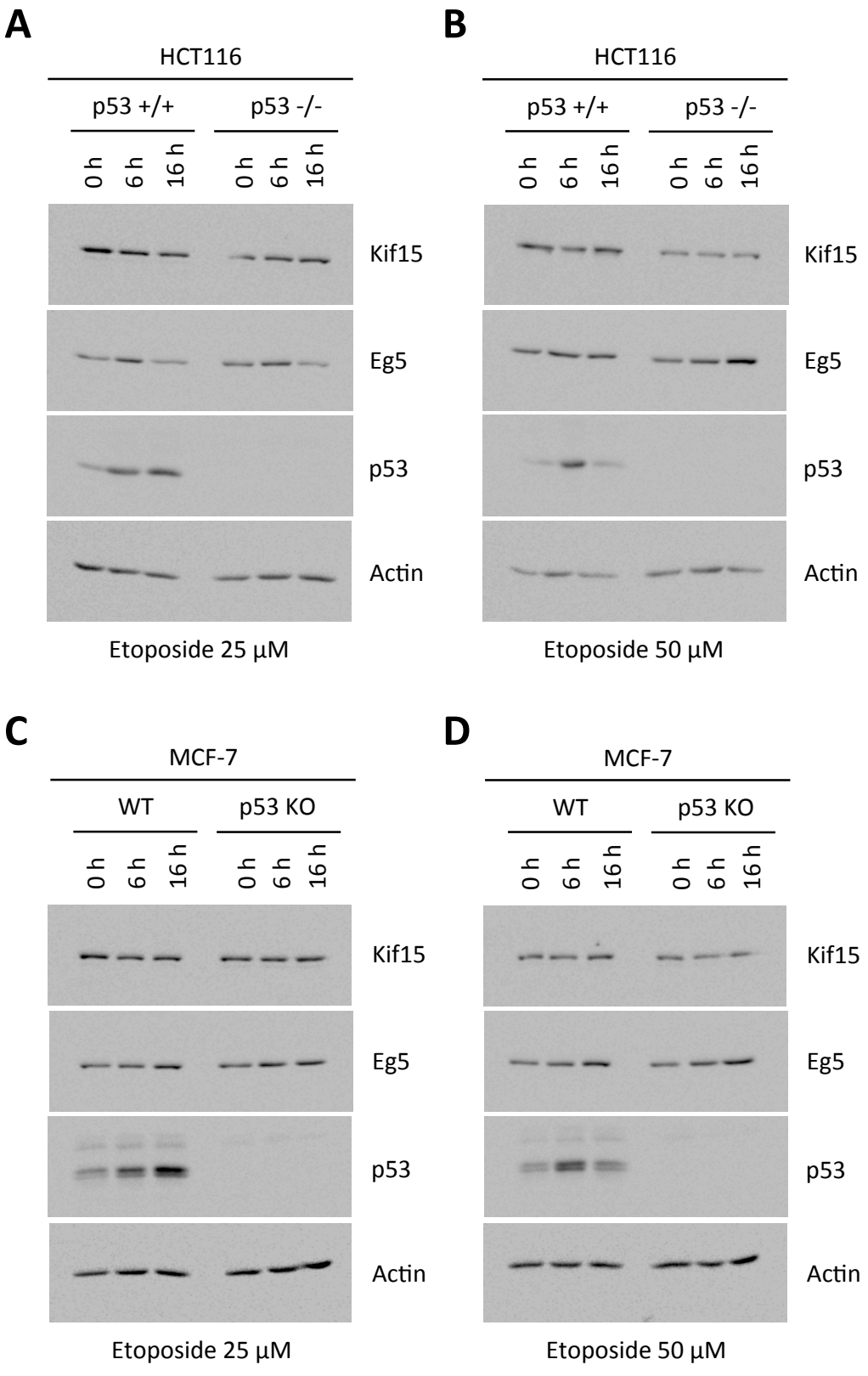
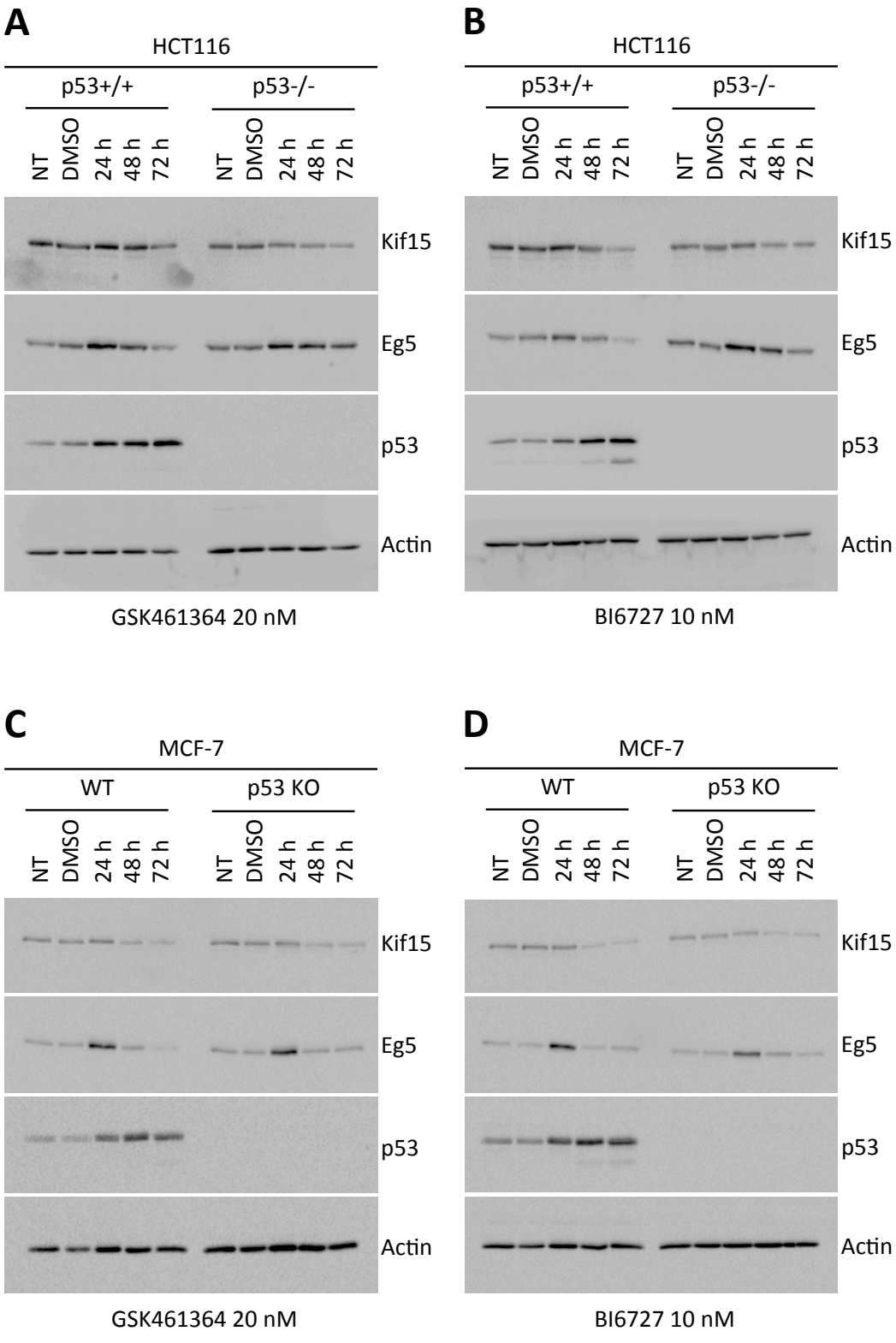


Figure 5.19. Kif15 and Eg5 levels upon treatment with etoposide. HCT116 p53+/+ and p53-/- (A+B) and MCF-7 wild type and p53 knockout cells (C+D) were treated with 25 µM (A+C) or 50 µM (B+D) etoposide. Cells were then harvested and analysed by western blotting with the antibodies indicated in the figure.


A
HCT116
p53+/+
p53-/-
NT
DMSO
24 h
48 h
72 h
Kif15
Eg5
p53
Actin
GSK461364 20 nM
B
HCT116
p53+/+
p53-/-
Kif15
Eg5
p53
Actin
BI6727 10 nM
C
MCF-7
WT
p53 KO
Kif15
Eg5
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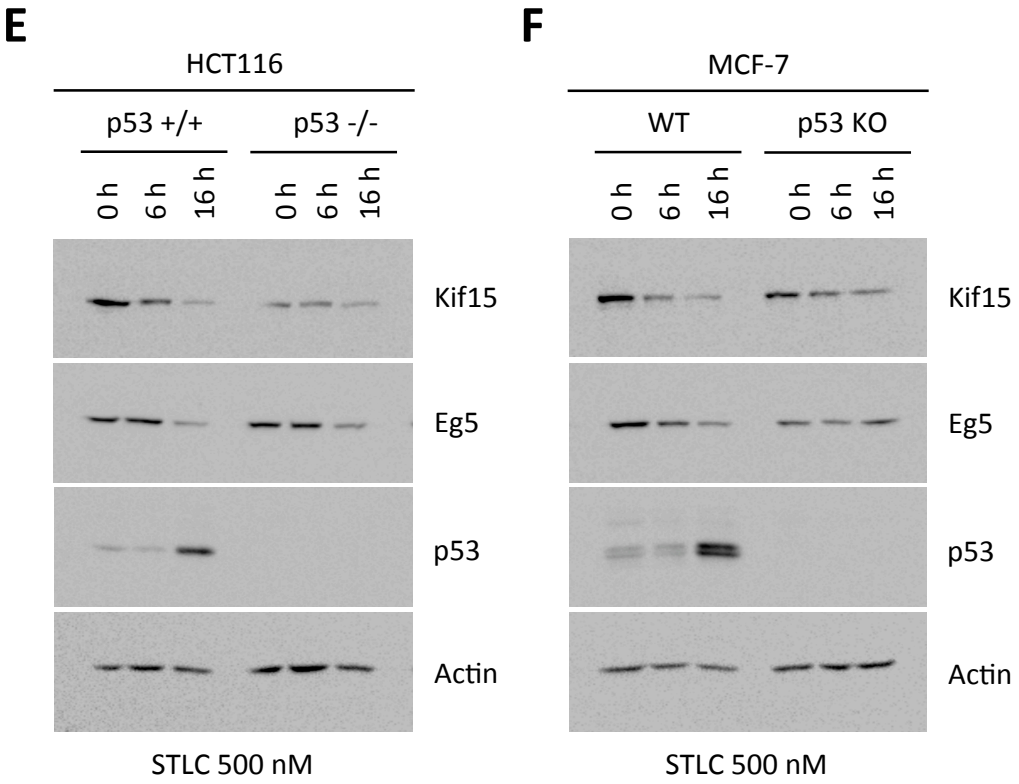

Figure 5.20. Kif15 and Eg5 levels upon treatment with PLK1 and Eg5 inhibitors. HCT116 p53+/+ and p53-/- cells (A,B+E) and parental MCF-7 and p53 knockout cells (C,D+F) were treated with PLK1 inhibitors, GSK461364 (A+B) and BI6727 (C+D), and Eg5 inhibitor, STLC (E+F). PLK1 inhibitors were applied for 24, 48 or 72 hours, along with an untreated and DMSO treated sample, whilst STLC was applied for 0, 6 or 16 hours. After harvesting, cell lysates were analysed by western blotting using the antibodies indicated in the figure.

5.4. Discussion

The findings of this chapter firstly show that upon treatment with PLK1 inhibitors the p53 dependent subset of cells that accumulate in G1 result from cells completing mitosis. Use of cell synchronisation by serum starvation and flow cytometry proved useful in allowing determination of this finding. Ideally, other methods of synchronisation would be tested to confirm this result. Further investigations show that in p53 wild type cell lines the delay in mitosis is shorter upon application of PLK1 inhibitors than in p53 deficient cell lines. This appears to be dependent on the DNA damage response pathway proteins ATM and ATR. As previously mentioned, ATM and ATR phosphorylate the serine 15 residue of p53 in response to cellular stress, such as DNA damage. Serine 15 has been shown to be a critical residue of p53 for its activation as a transcription factor (Loughery *et al.*, 2014). Therefore, activation of p53 appears to be required for the response to PLK1 inhibitors. The differences in mitotic duration cannot be accounted for by a p53 dependent delay in mitotic entry. As p53 can induce a G2/M arrest in response to DNA damage, and as it was observed that PLK1 inhibitors led to DNA damage, a delay in mitotic entry seemed a plausible mechanism. If p53 could delay entry into mitosis upon inhibition of PLK1, it may have allowed p53 to carry out transcriptional functions that may have been able to put a safety net in place before cells entered mitosis, where transcription is shut down (Gottesfeld and Forbes, 1997). This could have resulted in the observation of p53 competent cells having a higher success rate of completing cytokinesis in the presence of PLK1 inhibitors than p53 deficient cells. However, the experiments completed here suggest that is not the case. Additionally, the same effect is not observed when PLK1 inhibitors are replaced with Taxol, therefore suggesting that p53 does not have an effect once the spindle is established. Taken together, this implicates a role for p53 in early mitosis.

A p53-dependent difference in the activity of PLK1 can also not be attributed to the effects observed. By arresting cells in mitosis with Nocodazole and then applying increasing doses of PLK1 inhibitor, the activity of PLK1 could be determined by observing a PLK1 target. Phosphorylation of BubR1 at threonine 680 was used as a PLK1 target (Suijkerbuijk *et al.*, 2012). However, phosphorylation at this site showed no significant difference in activity of PLK1 between p53 competent or deficient cells. Therefore, it seemed unlikely that the mitotic effects observed were simply due to higher levels of activity of PLK1 in p53 competent cells leading to the same dose of PLK1 inhibitor having less effect on the p53 wild type lines.

The results presented here also show that p53 promotes centrosome separation when PLK1 is inhibited, due to the observation that a p53 wild type line has a greater proportion of separated centrosomes than a p53 deficient line when treated with GSK461364. Furthermore, inhibiting a downstream target of PLK1 that is involved in centrosome separation, the motor protein Eg5, results in a similar p53 dependent phenotype as inhibition of PLK1. This is the case in regards to the mitotic duration observed under the inhibition of Eg5 and the sensitivity to Eg5 inhibition.

The mechanism by which p53 exerts these effects is currently unknown. One hypothesis is that p53 regulates the levels of Eg5 or a functionally redundant motor protein, Kif15. As Kif15 has previously been shown to substitute for loss of Eg5 (Tanenbaum *et al.*, 2009), in maintaining efficient centrosome separation, this seemed a plausible idea. However, experimental procedures indicated that neither Eg5 nor Kif15 proteins were p53 inducible. Treatment with Nutlin-3 (allowing activation of p53 without DNA damage) and etoposide (resulting in p53 activation by DNA damage) both failed to produce p53 dependent effects in regards to the Eg5 or Kif15 levels. Similarly, treatment with PLK1 inhibitors or an Eg5 inhibitor, where the p53-dependent effects are

actually observed, also failed to result in any Eg5 or Kif15 differences. With thorough testing through use of several types of p53 activators, it could be concluded that Eg5 and Kif15 are not direct transcriptional targets of p53. However, activity of these proteins, or differences in their regulators and partners was not tested, which may prove useful to further investigate this hypothesis.

Chapter 6 : Conclusions and Future Perspectives

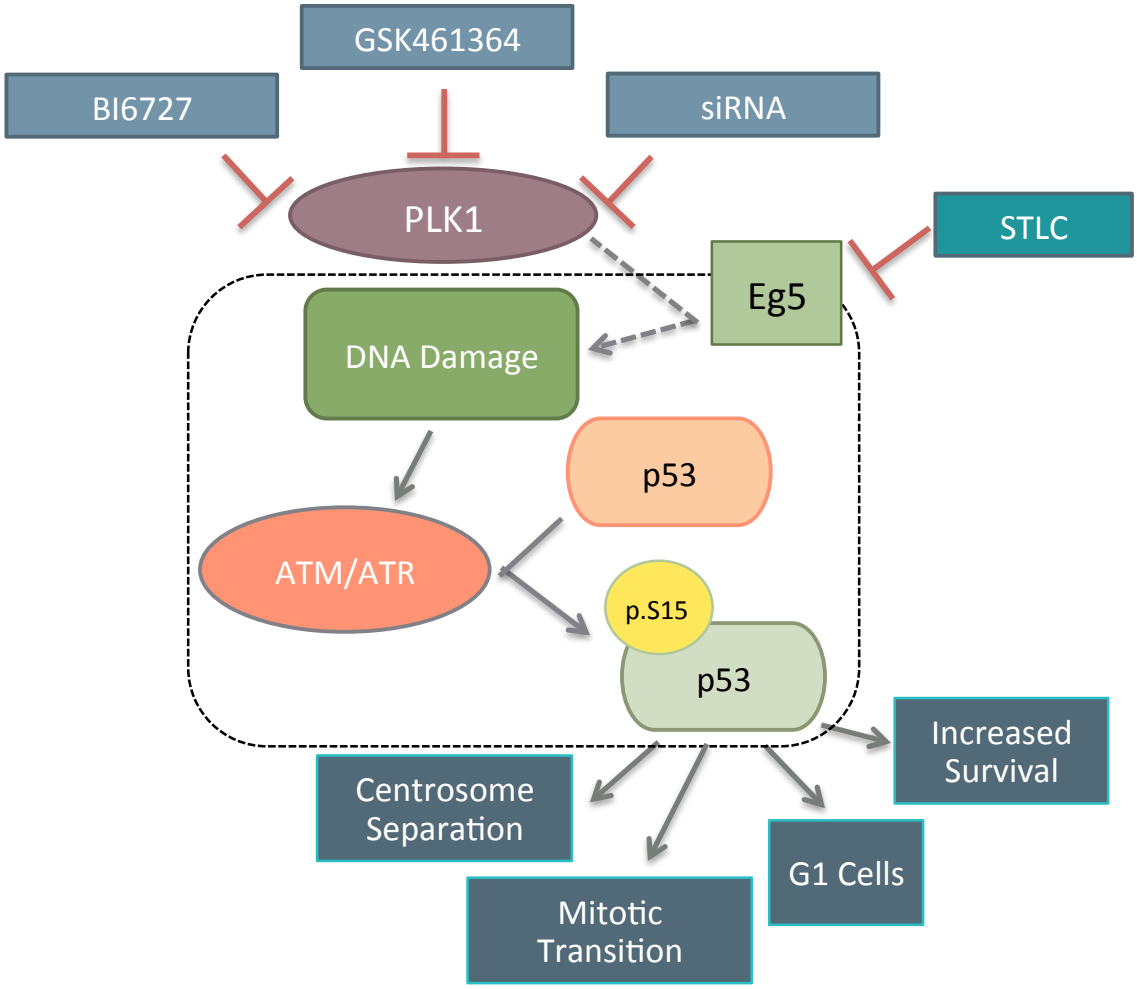

Figure 6.1. Conclusion summary. The overall findings of this thesis are represented in the schematic. As PLK1 is involved in a phosphorylation cascade that leads to activation of Eg5, inhibition of PLK1 or Eg5 show similar outcomes. Their inhibition or silencing leads to DNA damage, which results in activation of p53 through phosphorylation of the serine 15 residue of p53 by the DNA damage response pathway proteins, ATM and ATR. The presence of p53, when PLK1 or Eg5 is inhibited, leads to improved centrosome separation and a mitotic transition more comparable to the homeostatic normal, compared to the absence of p53. This results in an increase in the number of cells completing cytokinesis and returning to G1 with 2N DNA content, and overall results in an increased survival and decreased sensitivity to PLK1 and Eg5 inhibitors in comparison to the absence of p53.

The overall findings and conclusions of this thesis are represented in Figure 6.1. The findings support the findings of previous studies that show a reduced sensitivity of PLK1 inhibitors to cancer cells expressing wild type p53 (Guan *et al.*, 2005; Liu, Lei and Erikson, 2006; Sur *et al.*, 2009; Degenhardt *et al.*, 2010; McKenzie *et al.*, 2010; Danovi *et al.*, 2013; Yim and Erikson, 2014). Furthermore, the findings show that PLK1 inhibitors mainly arrest cancer cells in mitosis, result in DNA damage, and lead to cancer cell death or mitotic slippage, again supporting previous studies (Hyungshin Yim and Erikson, 2009; Driscoll *et al.*, 2014). However in addition to this, the results presented in this thesis show that a greater proportion of p53 competent cells can successfully complete cytokinesis in the presence of PLK1 inhibitors, than p53 null cells treated with the same dose. This adds to a previous study, which showed inhibition of Aurora A, an upstream activator of PLK1, resulted in an increased delay in mitotic exit in p53 deficient lines compared to p53 wild type cells (Marxer *et al.*, 2014). The results shown here further these findings, by showing these effects appear to be ATM and ATR dependent, as well as p53-dependent, suggesting that activation of p53 is required for this effect to occur. Inhibition of ATM and ATR, with small molecule inhibitors KU-55933 and VE-821, resulted in a reduction in the levels of p53 and phosphorylated p53 at residue serine 15, and resulted in a further increase in mitotic duration upon treatment with PLK1 inhibitors, therefore ablating the protective effect. The activation of p53 could be therefore be through the DNA damage response pathways in response to the DNA damage observed by inhibition of PLK1 or this effect may be due to basal levels of p53 activity. Previous work in the Meek lab showed that ATM and ATR maintain a low basal level of p53 serine 15 phosphorylation in cultured cancer cells that is required for p53 dependent transactivation in the absence of exogenous cell stress, such as DNA damaging agents (Loughery *et al.*, 2014). Therefore it is currently unknown whether an activating stimulus, such as the PLK1 inhibitor

induced DNA damage, is required for the p53-dependent activation and effects or if the basal levels of phosphorylated p53 alone are sufficient.

The results shown here further explore the roles of p53, by honing in on the action of p53 in mitosis. The experimental procedures utilised, whilst not confirming the full mechanism, have narrowed the areas in which p53 can act. By showing that p53 does not delay mitotic entry, and the fact that interfering with the depolymerisation of microtubules (through use of Taxol) does not result in a p53 dependent outcome, the role of p53 has been implicated in early mitosis. Due to PLK1 being involved in centrosome separation during early mitosis, this aspect of its function was warranted for investigation. As a result of this investigation the data presented in this thesis also shows a potential novel role for p53 in the separation of the centrosomes. Previous studies have suggested a role for p53 in centrosome integrity. However, this has been suggested to be through p53 repressing the stimulation of centrosome separation by Cyclin B2, therefore, resulting in a p53-dependent down-regulation of centrosome separation. Cyclin B2 overexpression was shown to induce hyperactivation of PLK1 through Aurora A, thus resulting in an acceleration of centrosome separation. The same study showed that p53 acted antagonistically to Cyclin B2 in normal cells, by controlling Aurora A (Nam and van Deursen, 2014). The results shown in this thesis suggest that there is another mechanism in this pathway in which p53 can actually stimulate the separation of centrosomes when the pathway involved in stimulation of centrosome separation is inhibited. To further this work, the mechanism by which p53 has such an effect on the centrosome separation pathway should be investigated.

How p53 would exert such a role in mitosis is currently unknown. Although Cyclin B1 was shown to be transcribed during mitosis in Hela cells (Sciortino et al. 2001), it is generally accepted that transcription shuts down during mitosis. This is due to the chromatin being highly condensed, so transcription is near impossible as promoters are

inaccessible and specific RNA polymerase II factors are displaced from the chromosomes (Gottesfeld & Forbes 1997). Based on this, it seems unlikely that p53 would be able to transcriptionally activate the expression of target genes during mitosis. Therefore, p53 may be able to carry out a transcription-independent function in mitosis in response to centrosomal problems, or there may be a p53-dependent compensatory pathway that comes into play when mitotic problems are encountered.

Eg5 is the motor protein that has been shown to drive the centrosomes apart in nearly all tested organisms (Ferenz, Gable and Wadsworth, 2010). However, in the event of inhibition of Eg5, Kif15 has been shown to substitute for the lack of Eg5 (Tanenbaum *et al.*, 2009). As inhibition of Eg5 by STLC resulted in a similar p53 protective effect to that of inhibition of PLK1, the p53 protective effect may have occurred through either Eg5 or Kif15 levels being stimulated by p53. However, preliminary data suggested that neither Eg5 nor Kif15, two motor proteins involved in the separation of centrosomes, are direct transcriptional targets of p53. Treatments with Nutlin-3, etoposide, PLK1 inhibitors (GSK461364 or BI6727) or Eg5 inhibitor (STLC), did not result in a p53-dependent change in the total levels of Eg5 or Kif15 proteins. This suggested that the levels of these motor proteins are not affected by p53, although for a more thorough investigation the activity and localisation of these proteins could also be tested, to determine if either protein was more active in p53 competent cells or if p53-dependent differences in localisation occured. Similarly, whilst Eg5 and Kif15 may not be direct transcriptional targets of p53, a compensatory effect may involve p53-dependent regulation of regulators or partners of these proteins. Additionally, dynein, a third motor protein that is also involved in centrosomal forces, could be a potential p53 target candidate. Studies have suggested that all three motor proteins are required to act together to generate the correct force balance in the spindle (vanHeesbeen, Tanenbaum

and Medema, 2014). Therefore, future work could investigate if dynein is a transcriptional target of p53.

In regards to the sensitivity differences observed that are dependent on the p53 status of cancer cells, further work could be carried out. As this has only been shown in cell lines it would be interesting to test if this holds true *in vivo*. There has been a wealth of clinical data generated through the clinical trials testing PLK1 inhibitors. One clinical trial study showed that the response to BI6727 was independent of the genetic subgroup (Döhner *et al.*, 2014). However, p53 status was not one of the factors that was assessed. Access to the patient data would be very beneficial to assess whether there is an association between responders versus non-responders in regards to their p53 status.

Determination of the surviving cells would also assist in improving treatment options for patients. The data here indicate that there is a subpopulation of cells that manage to complete mitosis successfully, to return to G1, in a p53-dependent manner. These cells are the most plausible source of survival upon treatment with PLK1 inhibitors. However, this has not been proven. By sorting the treated cells, perhaps this could be tested. By knowing what cells are the source of survival, and thus resistant to these inhibitors, a targeted combination treatment could be used. Different chemotherapeutic drugs are effective in different stages of the cell cycle. Therefore, knowing where the surviving cells are likely to be in the cell cycle may assist in improving efficacy of PLK1 inhibitors in combination with another chemotherapeutic agent.

The later part of this research suggested that effects observed with PLK1 inhibitors were also observed with Eg5 inhibition. This would indicate that the same pathway is affected by both these types of inhibitors. This could then suggest that assessment of patients for their p53 status may not only be beneficial for PLK1 inhibition, but also for Eg5 inhibition. In order to further test this, combining a PLK1 inhibitor with an Eg5

inhibitor, and looking for any additive effects, would allow determination of whether only the same pathway was affected. Any inhibitors of other proteins involved in centrosome separation could also be tested for similar p53-dependent effects. As only Eg5 was investigated, it would be useful to determine if similar effects are observed with depletion of proteins involved in the centrosome disjunction pathway. Overall this could prove to be very important information to obtain, as it could impact on the treatment of patients. A better understanding of how the p53 status and drugs directed at PLK1, and other proteins involved in the centrosome separation pathway, are related is required for ensuring the best treatment options are available to the diverse range of cancer patients requiring treatment.

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Chapter 8 : Appendix

The disc contains four live-cell imaging movies relating to Figure 5.8. HCT116 p53+/+ and p53-/- cells stained with SiR-DNA were imaged every four minutes over approximately 16 hours. The four movies included on the disc are:

1. HCT116 p53 +/+ cells treated with DMSO
2. HCT116 p53+/+ cells treated with 20 nM GSK461364
3. HCT116 p53-/- cells treated with DMSO
4. HCT116 p53-/- cells treated with 20 nM GSK461364.